

PHOTO- AND CHEMOTAXIS
IN ARCHAEBACTERIA:
FLAGELLAR APPARATUS, EXCITATION
SIGNAL TIMES AND TRANSDUCERS
OF HALOBACTERIUM HALOBIVM
AND HALOPHILIC SQUARE BACTERIA

Maqsudul Alam

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PHOTO- AND CHEMOTAXIS IN ARCHAEABACTERIA:
FLAGELLAR APPARATUS, EXCITATION SIGNAL TIMES AND TRANSDUCERS
OF *HALOBACTERIUM HALOBIVM* AND HALOPHILIC SQUARE BACTERIA

Dissertation
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2.2 Methods

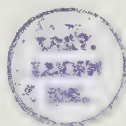
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SUMMARY

Halobacterium halobium has right-handed helical flagella. During the logarithmic phase of growth, cells are predominantly monopolar, whereas in the stationary phase they are mostly bipolarly flagellated. The flagellar bundle consists of several filaments. Halobacteria swim forward by clockwise and backward by counterclockwise rotation of their flagella. The flagellar bundle does not fly apart when the sense of rotation changes. In addition to the flagella attached to the cells, large amounts of loose flagella, which aggregate into thick super-flagella, can be observed at all phases of growth. During stationary phase, the production of these super-flagella, which are generally 10 to 20 times longer than cell body, is significantly higher. Flagellar filaments were purified by dissociation and reconstitution. Three different protein bands (23,500; 26,500 and 31,500 apparent molecular weight) are seen on sodium dodecyl sulphate/polyacrylamide gels, thus confirming that all three proteins are intrinsic to the flagellar structure and designated as flagellin Fla I (23,500), Fla II (26,500) and Fla III (31,500). Antibodies against halobacterial flagella were produced in chicken because antibodies of these birds interact with their antigens even in 4 M-NaCl. Rotation of tethered cells demonstrated that Halobacteria move due to the rotation of a flagellar motor. Polymorphic transitions from normal to a curly, a ring and a straight form were induced by different pH values and heat

treatments.

Halophilic square bacteria were shown to have also right-handed helical flagella. They swim forward by clockwise, and backwards by counterclockwise rotation of their flagella. The cells were propelled by several filaments or a single one arising at several or single points at the cell surface. Several filaments can form a stable bundle that does not fly apart during the change from clockwise to counterclockwise rotation or *vice versa*. In addition to the flagella attached to the cells, large amounts of detached flagella aggregated into thick super-flagella, can be observed at all phases of growth. In contrast to Halobacteria with three flagellins, square bacterial have only one flagellin with an apparent mol. wt. of 73 000.

Excitation signal processing times in halobacterial phototaxis were monitored with a computerized cell-tracking system coupled to an electronic shutter controlling delivery of photostimuli. Automated analysis of rates of changes in direction and linear speeds provided detection of reversals with 67 milliseconds resolution, and permitted measurement of the responses to attractant and repellent stimuli. After stimulation, a latency period was observed in which the reversal frequency was unchanged, followed by an excitation phase in which reversal frequency increased, and an

adaptation phase in which reversal frequency returned to its prestimulus value. A step-decrease in illumination of the attractant receptor sensory rhodopsin(SR) (λ_{max} , 587 nm) was interpreted by the cells as an unfavorable stimulus and, after a minimum latency of 0.70 ± 0.14 s, induced reversals with the peak response occurring 1.34 ± 0.07 s after the onset of the stimulus. Two distinct repellent responses in the near UV/blue were observed. (a) One was a reversal response to 400 nm light, which was dependent on orange-red background illumination as expected for the photointermediate repellent form of SR(λ_{max} , 373 nm). The minimum latency of this response was approximately the same as that of the SR attractant system. (b) The second was a reversal response with shorter minimum latency (0.40 ± 0.07 s) to light of longer wavelength (450 nm) than absorbed by the known SR repellent form.

Transducer or the so called methyl-accepting proteins in *Halobacterium halobium* appeared on SDS-polyacrylamide gel as a series of ^3H -methyl labeled bands with apparent mol. wt. in the region of 90-125 and 20-25 kilodaltons. These methyl-accepting proteins are membrane associated proteins and release of methyl groups by sodium hydroxide treatment demonstrated an ester linkage in analogy to the eubacterial transducers. *Halobacterial halobium* mutants defective in chemo- and phototaxis exhibit greatly reduced levels of methyl-labeled proteins. Addition of both attractants or repellents to the labeled wild type cells decreased the

extent of labeling of transducers. The same phenomenon was observed in chase experiments analyzing the activity of methylesterase in intact cells. The positive as well as negative chemical or photo stimuli caused a rapid production of methanol with gradual return to the prestimulus level.

INTRODUCTION

1.1 General background

Halobacterium halobium, a member of Archaeobacteria is a unique entity for studying sensory function in which sensory reception, signalling, adaptation and integration can be studied at both the chemo- and phototaxis level without interference of bioenergetic processes such as photosynthesis or respiration. During the last 15 years the study of phototaxis in halobacteria was dominated mainly by research on receptor and very little is known about flagella and transducers of Halobacteria. Nothing has so far been reported about the flagella and motility behaviour of halophilic square bacteria.

Bacterial chemotaxis is a transient response of an organism in a situation where environmental homogeneity has been disturbed by certain chemical compounds. Two analogies with higher eucaryotes sensory phenomena are revealed by the study of bacterial chemotaxis. First, bacterial chemotaxis is similar to the stimulus-response of neuronal, immune and sperm cells. Second, studies of individual components involved in the bacterial sensory pathway can contribute to the understanding of the function of receptors, controlling signals and molecular comparators in transmembrane signalling systems. This phenomenon of chemotaxis has been in details described in motile bacterial species including

enteric bacteria (Adler et al., 1975; Koshland, 1981 and Ordal et al., 1983), Sprichaetes (Goulbourne and Greenberg, 1983) and in short in Archaeobacteria (Hildebrand, 1977).

1.2 Chemotactic behaviour

In an homogeneous environment, *E. coli* alternates its swimming behaviour either "running" for a period of a few seconds or "tumbling" for a few tenths of a second which results in abrupt change of direction (Berg and Brown, 1972). The mechanical explanation is that the flagella, normally five to eight per cell, form a bundle to coordinate their counterclockwise rotation when the cell is running (Berg, 1974; Silverman and Simon, 1974). When the cell is tumbling individual flagella loose their coordination by changing their direction of rotation to clockwise.

Due to the transient nature of the chemotactic response, two distinguishable phases, excitation and adaptation can be observed (Hazelbauer and Harayama, 1983). For a brief period of time after stimulation, changes in swimming pattern occur (excitation), i.e. a rapid deviation from pre-stimulus tumbling probability arises. Positive stimuli decrease this probability while negative stimuli increase the tumbling frequency. This deviation is progressively recovered in the adaptation phase by a restoration of that probability. Consequently, all types of enviromental stimuli are

internally processed to modulate a binary mode of flagella rotation.

1.3 Flagella

The external locomotive organelles of most bacteria are quite similar as all are built from a single protein and have the shape of a hollow, spiral cylinder. The entire flagellum consists of a filament, a basal body located in the cell envelope, and a short hook structure that links these two parts. The filament is usually longer than the cell body, being 10-15 μm long on a 2 μm -long *E. coli* cell. The molecular weight of flagellins varies greatly among bacteria, from 25,000 to 63,000 and the fine structure of the filament is rather complex. Electron microscopy has revealed that the filament is a cylinder having 150-200 Angstrom diameter with a wall composed of 11 helically wrapped flagellin strands (Finch, Klug, 1972). The filaments can be dissociated to monomeric protein, and reconstituted *in vitro*, without enzymatic mediation, to give filaments indistinguishable from originals by all known criteria (Asakura et al., 1966). A variety of packing arrangements ("polymorphs") for flagellin is known. "Curly", "straight", "coiled" and other discrete structures have been produced in various ways, by (1) mutation (Iino, 1962; Iino & Mitani, 1967) in some instances involving only a single amino acid substitution (2) incorporation of amino acid

analogues into flagellin (3) changes in environmental parameters such as pH and ionic strength (Shimada et al., 1975; Kamiya & Asakura, 1976); (4) copolymerization of mixed monomers (Asakura & Iino., 1972).

1.4 Genetics and components of information processing

Genetic studies have revealed that about 50 different gene products are involved in chemotactic behaviour (Parkinson and Hazelbauer, 1983). The functions of the gene products can be classified as chemoreception, sensory transduction, control of swimming pattern and motility. Cytoplasmic location of most *che* gene products (Ridgeway et al., 1977) and their mutant phenotypes defective in control function suggest that signal processing in chemotactic response occurs primarily in the cytoplasm. The phenotypes of the *che* mutants are either counterclockwise (CCW) or clockwise (CW) biased (Parkinson, 1981). Since direct interactions between transmembrane receptors and motor components composed of *fla* and *mot* gene products are not likely to occur (Engstrom and Hazelbauer, 1982; Boyd and Simon, 1983) the *che* gene products should mediate between the receptors and the motor. The roles of these intermediate signalling components have been assessed from their null mutant phenotypes. Two *che* mutants, *cheB* and *cheZ*, produce CW bias but still exhibit partial responses to positive stimuli (Parkinson, 1981). Three other *che* mutants, *cheA*,

cheR and *cheW* have CCW bias but respond to repellent stimuli (Kihara and Macnab, 1981). However, *cheY* mutants produce CCW bias and lack all sensory responses to both attractants and repellents (Clegg and Koshland, 1984), implying that this gene product is directly involved in receptor-motor linkage while others may have indirect modulatory roles in signal processing. Failures to identify signal candidates, for example, membrane potential change (Margolin and Eisenbach, 1984), Calcium ion (Snyder et al., 1981; Eisenbach, 1985;) and cGMP or cAMP (Taylor, 1985) further support direct involvement of *cheY* protein in signalling, possibly through a protein relay system. It has been demonstrated that signal propagation occurs at a slow rate, which also reduces the possibility of small molecules as the signal (Segall et al., 1985). Based on genetic evidence two independent feedback controls of sensory adaptation involving *cheR-cheY* and *cheB-cheZ* interaction, respectively, have been proposed (DeFranco et al., 1979; Parkinson, 1981). In addition, the *cheY* and *cheZ* proteins appear to interact with the components of the switch complex involved in flagellar rotation (Parkinson and Houts, 1982). *cheY* protein interaction with the switch-motor complex has also been demonstrated in vitro (Ravid and Eisenbach, 1984).

1.5 Transducers: Multifunctional sensory Proteins

Transducers are transmembrane receptor proteins located

in the cytoplasmic membrane of enteric bacteria (Hazelbauer and Harayma, 1983). Four transducers have been identified: the Tsr transducer binds serine (Hedblom and Adler, 1980); the Tar protein binds aspartate as well as maltose-binding protein (Koiwai and Hayashi, 1979); the Trg interacts with ribose-binding protein and galactose-binding protein and the Tap binds small peptides (Manson et al., 1986). Binding of ligand to the transducer at a site exposed to the external face of the cytoplasmic membrane results in a drastic shift in the bias of the flagellar motor. Behavioural adaptation to positive stimuli is linked to carboxyl methylation of specific glutamyl residues in the transducer protein through which excitation is mediated. Adaptation to the negative stimuli is linked to demethylation. Multiple sites for these covalent modifications permit graded levels of adaptation. Thus the extent of covalent modification of a population of transducer molecules reflects the extent of occupancy of the ligand-binding site a few moments previously. Methylation and demethylation of transducers has the features of a rudimentary "memory" exhibited by the sensory system. Three functional demands can be defined: ligand-binding, excitation and covalent modification for adaptation. The primary structure of transducers have been derived from their amino acid sequence and allow topographic models of the molecules with respect to their extracellular, intracellular and transmembrane domains. The conclusions were derived from biochemical characterization of the sites of covalent modification (Kehry et al 1982a, 1982b, 1983a,

and 1985) The extent of homology among the carboxyl-terminal domains of the four transducers is striking. The extensive homology clearly suggests that the nucleotide sequences corresponding to the carboxyl-terminal domains are all derived from a common ancestral sequence (Bollinger et al, 1985).

1.6 Halo- and square bacteria

The extremely halophilic microorganism *Halobacterium halobium* belongs to the kingdom of archebacteria. Rather interestingly, it contains several retinal-protein complexes. Until their discovery the only known retinal proteins were the visual pigments of animals. The retinal-protein first discovered in *Halobacterium halobium* was bacteriorhodopsin which acts as an outwardly directed light-driven proton pump (Oesterhelt & Stoeckenius, 1971; 1973; Oesterhelt, 1972;). A second retinal-protein halorhodopsin acts as an inwardly directed light-driven chloride pump (Mukohata et al., 1980; Schobert & Lanyi, 1982;). Two other retinal-proteins: sensory rhodopsin and protein P₄₈₀ mediate "colour" vision that aids the cell in finding the optimal photosynthetic environment (Spudich & Bogomolni, 1984; Takahashi et al., 1985).

According to Hildebrand and colleagues these bacteria contain two retinal-based pigments which serve to detect changes in light-intensity and control behaviour (Dencher & Hildebrand, 1979; Hildebrand, 1983; Hildebrand & Schimz,

1983; Sperling & Schimz, 1980). These two systems should, however, include in some way the retinal protein mentioned above. *Halobacterium halobium* cells are attracted by D-glucose and several amino acids and are repelled by phenol. Certain chemicals such as acetate, benzoate, indole and NiSO_4 , that are known to act as repellents of *Escherichia coli* cells, attract Halobacteria (Schimz & Hildebrand, 1979). Preliminary reports provided evidence for methyl-accepting proteins involved in photo- and chemotaxis (Schimz., 1981, 1982; Bibikov et al., 1982). Early investigators reported that *Halobacterium halobium* is only monopolarly flagellated (Houwink, 1956), whereas later studies suggested that they are only bipolarly flagellated (Hildebrand, 1977). In addition to the flagella attached to cells, fully grown cultures also contain large amounts of loose flagella, sometimes aggregated into thick spiral bundles (Dundas, 1977).

Observation of samples taken from the brine of a salt pond in Southern Sinai revealed a new morphological type of bacterium with a unique square rectangular shape (Walsby, 1980). A number of isolates of square-shaped bacteria exhibit the general characteristics of extreme halophiles and archaeobacteria (Kessel and Cohen, 1982; Stoeckenius, 1982; Javor et al., 1982).

I want to ascertain whether *Halobacterium halobium* and the halophilic square bacterium have the same flagellar

apparatus, and to transducer like proteins as in eubacteria, and to further characterize them in relation to chemo- and phototaxis.

C: MATERIALS AND METHODS

1.1 Materials

1.1.1 Chemicals

Bacto-Pepton and Tryptone (Oxoid) used in growth media were from Difco, Detroit, Michigan, USA. Compounds used as attractants and repellents in assays of chemotaxis were of the highest purity from Sigma Chemical Co.

L-(methyl-³H) -methionine (10 to 15 Ci/mmol or 65 to 80 Ci/mmol) was purchased from Amersham Co., USA. All other chemicals were reagent grade.

1.1.2 Bacterial strains

	SOP P ₄₈₀		Ret	Pho	Che	Literature
H.halobium S9	+	+	+	+	+	Stoeckenius, 1979
H.halobium L33	+	+	+	+	+	Wagner, 1981
H.halobium M175	+	+	+	+	+	Lab collection
H.halobium Flx3	+	+	+	+	+	Bogomolni, 1982
H.halobium M402	+	+	+	+	+	Lab collection
H.halobium L07	?	?	-	-	+	Lab collection
H.halobium Flx15	+	+	+	+	+	Spudich, 1985
H.halobium Pho81	-	-	-	-	+	Sundberg, 1986
Square bacteria						
isolate 801030/1	ND	ND	ND	+	+	Kessel, 1982

1.2 Methods

1.2.1 Growth Medium and selection of highly motile cells

Complex medium (Oosterhelt and Stoeckenius, 1974)

NaCl	250 g
MgSO ₄	20 g
Na ₃ Citrate	3 g
KCl	2 g
Oxoid-Peptone Code L 37	10 g
glass-distilled H ₂ O	1000 ml; pH 7.2

Agar medium

Complex medium plus 0.25% or 1.5% Bacto Agar

Basal salt medium

Complex medium without Peptone

Wash medium

Basal salt medium plus 0.1% Arginine

Chase medium

Wash medium	500 ml
L-methionine(10 mg/ml stock solution)	750 μ l

Attractant and Repellent Solutions

Solutions were prepared in Chase medium

Selection of highly motile cells

A culture grown in complex medium was inoculated into the center of a soft agar(0.25%) plate. After three to five days of incubation at 37°C an annulus of high cell density (a "chemotactic ring"(Adler, 1966)) of about 2 to 3 cm diameter formed around the center. Cells were inoculated from this outer ring and inoculated into a freshly prepared soft agar plate. Cells from the chemotactic outer ring of the second plate were streaked out and grown in complex liquid medium to the middle of the exponential phase, screened microscopically for motility, and the culture of qualitatively best motility was selected for further chemo- and phototaxis experiments.

1.2.2 Behavioral observations

Cells from late exponential phase cultures were diluted 100- to 200- fold in fresh growth medium and incubated for 1-2 hour before use in behavioural experiments. The cells were kept at approximately at 37°C by means of a water-jacketed slide holder on the microscope stage, observed in the dark-field with infrared light($\lambda > 700$ nm) provided by a 12-V, 100-W tungsten-halogen lamp and infrared transmittance filters (7-69; Corning Glass Works, Corning, NY), and monitored by an infrared-sensitive video camera (Newvicon; 1 in.;RCA New Products Div., Lancaster,PA) mounted on the microscope. Light stimuli were provided by 150-W Xenon arc lamps, or by a 200-W Mercury arc lamp, the

beams from which were combined with the monitoring beam using a cold (visible-reflecting, infrared-transmitting) mirror and beamsplitter (Edmund Scientific Co., Barrington, NJ) before entering the microscope optics. A 600 ± 20 nm interference filter was used for exciting the attractant photosystem, a 520 nm short-pass and 400 ± 5 nm or 450 ± 20 nm interference filters were used for exciting the repellent photosystems (all from Ditric Optics, Inc., Hudson, MA). Delivery of light stimuli was controlled by means of an electronic shutter (Vincent Associates, Rochester, NY) coupled to the digitizing unit of the automated tracking system (Motion Analysis Corp., Santa Rosa, CA) via a pulse generator (Grass Instrument Co., Quincy, MA) that controlled the delay between an event marker used to initiate data acquisition by the computer and delivery of the stimulus.

1.2.3 Visualization of flagella by Dark-field Microscopy and video recording

Dark-field microscopy was carried out according to Macnab (1976) with some modification. The Leitz Universal Orthoplan microscope was equipped with Leitz oil immersion dark-field condenser (numerical aperture 1.2/1.4); Leitz NPL 50x and 100x objective (numerical aperture 1.32/0.6) and Periplan CW 10xM eyepieces.

The movement of cells with rotating flagellar bundles was recorded with an ultrasensitive high-resolution video camera (Newvicon WV-1850/G), video recorder (Sony U-matic VO-5360).

and high resolution video monitor (Panasonic WV-5410). For calculation of speed an electronic microsecond timer (Panasonic time-date generator WJ-840) was used. All observations were carried out at $38 \pm 2^{\circ}\text{C}$ using an automatic thermoregulated stage (Leitz, type 050-117,004). The light source for dark-field microscopy was a Xenon high-pressure lamp (450 W). The images of moving square bacterial cells with rotating flagella were recorded with an extra 2x lens adjusted to the video camera tube.

1.2.4 Preparation of slides

Because of the very high light intensity used, particular attention was paid to cleanliness of the slides. Microscopic slides (0.72 mm thickness) and cover slips (0.15 mm: 50 ELKA Menzel-Glaser, F.R.G.) were immersed in chromic acid for two days, rinsed several times with distilled water and dried with a laboratory dryer. A 5 μl aliquot of sample was about the right amount.

1.2.5 Instant photography

A Vario Orthomat camera with an automatic microphotographic system was used. Micrographs were taken at different magnifications and with different exposure times using Ilford HP5 (35 mm ASA 400) and Kodak recording films 2475 RE 135-36 (ASA 1000). The films were processed to an equivalent speed of ASA 3600 with Ilford Microphen Developer-Replenisher

for 16 min at 20°C.

1.2.6 Analysis of behavioural data

Automated tracking of swimming *H.halobium* and measurement of light-induced behavioural responses using a computer-linked system has been described earlier by Takahashi and Kobatake(1985). We used a commercially available system recently introduced by Motion Analysis Corporation (MAC).

All of the analysis has been carried out using appropriate combinations of the operators defined in the MAC software package. The first stage of analysis was the reduction of video data to generate two-dimensional projections of the cells swimming paths. Video images were digitized frame-by-frame and transferred to the computer, where a combination of searching operations tracks cells from one frame to the next. The digitizing and search operations require input in the form of user-specified parameters such as frame rate, time interval, and search mask sizes, all of which have been incorporated into a user program, which allows one to automate the process. The version of the program used in the experiments digitized 5 seconds segments of video data at 15 frames per second, which gives a maximal time resolution of 67 milliseconds. The program was set up to carry out path calculations on 40 segments of digitized data, followed by merging of the individual path files to form a single file containing information on hundreds of paths.

Examples of paths generated by the program are shown in Fig.1. The second stage of the analysis makes use of motility parameter calculations to determine if and when reversals occur in a given path, followed by an ensemble summation over the entire collection of paths to determine the total number of reversals occurring at each time point. Here we are using two parameters to identify reversals: the rate-of-change-of-direction(RCDI), which reaches a maximum value when a cell undergoes reversal, and the "swimming speed, which passes through a minimum value during reversal. The basic strategy is as follows. RCDI values are calculated for each point along each path, as illustrated in fig. 2A, followed by application of interval-delta and local-maximum operators to convert the RCDI functions to binary functions. The interval-delta operator is used to set a threshold that distinguishes between RCDI values corresponding to reversals and background fluctuations. The result, as shown fig. 2C, is a step-function having a value of unity wherever the RCDI value is above threshold. Similarly, the local- maximum operator picks out each maximum value in the RCDI plot, generating the spike-function shown in fig. 2B. Multiplying the local- maximum and interval-delta functions together generates a function that has a value of unity wherever the original RCDI function had a maximum value above threshold (Fig. 2D). A similar operation is carried out on the swimming speed function and multiplied with the above to correlate minimum speed with maximum RCDI, our operational definition of reversal. Summing this function over time yields the

number of reversals occurring in each path during the analyzed time interval(5 seconds).

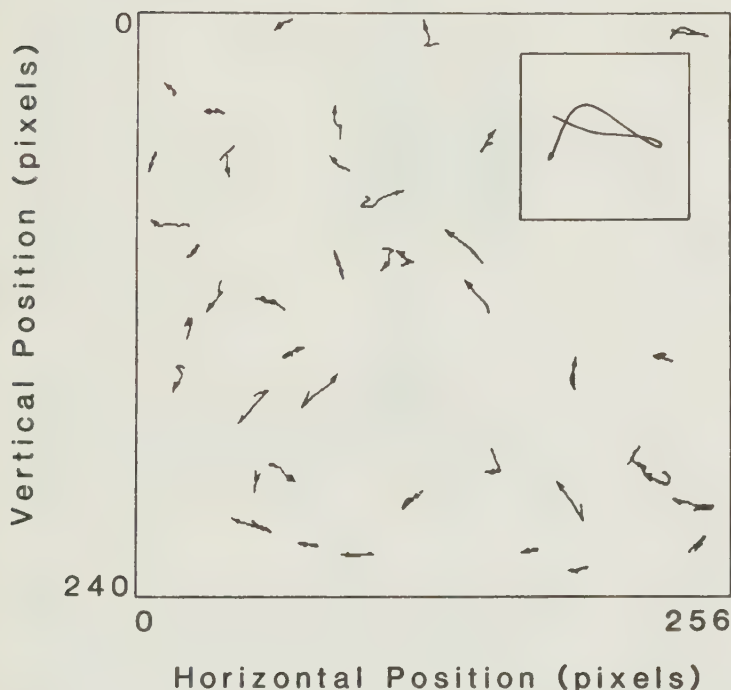


Figure 1:

Example of paths generated by motion analysis system. The paths were computed from digitized video images of *H. halobium* mutant Flx15. A series of search operations identifies the number of cells in each video frame, calculates a center-of-mass (centroid) for each object, and tracks the centroids from one frame to the next, thereby generating two-dimensional projections of the cell's swimming trajectories. The paths have been smoothed to minimize small variations in the positions of centroids resulting from the slow translational motion of the cells relative to fluctuations of their digitized outlines. Data were collected at 37°C for 5 s, and processed at 15 video frames per second. A repellent stimulus was delivered 1 s after initiation of data collection. Inset: enlarged view of a single path.

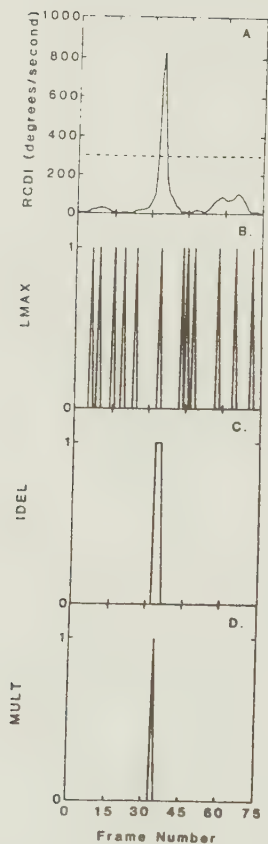


Figure 2:

Illustration of the strategy used to identify if and when a reversal of swimming direction occurs in a given path. The analysis makes use of appropriate combinations of the operators defined by the MAC software to detect abrupt changes of swimming direction. (A) The rate-of-change-of-direction (RCDI) function calculated for the path shown in the inset of Fig. 1. The dotted line indicates a threshold used to discriminate between values of the RCDI function representing reversals from background fluctuations. (B) Binary function resulting from application of the local-maximum operator (LMAX) to the RCDI function. (C) Binary function resulting from application of the interval-delta operator (IDEL) to the RCDI function using the threshold shown in (A). (D) Multiplication of (B) and (C) results in a function having a single spike wherever the original RCDI had a maximum value above threshold. A similar treatment of the swimming speed function is carried out and used to correlate maximum rate-of-change-of-direction with minimum velocity.

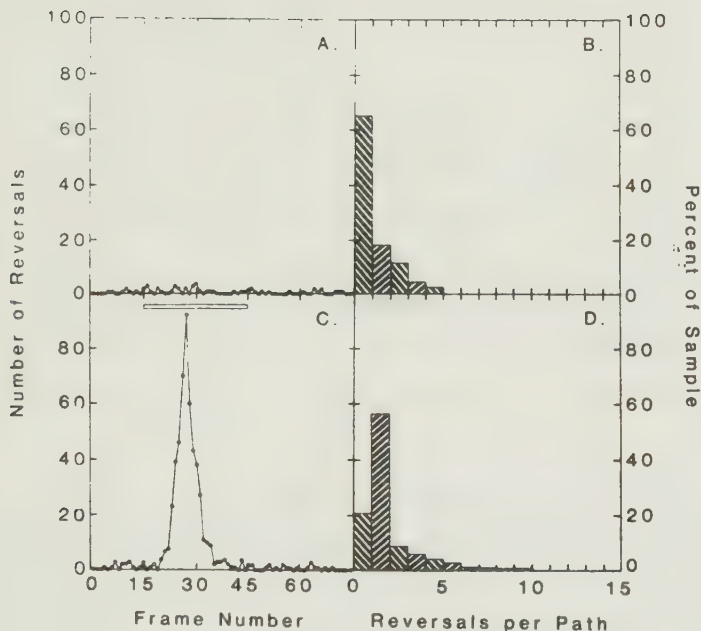


Figure 3:

Reversal distributions and corresponding histograms of the number of reversals per path. (A) Nonstimulated cells. The reversal distribution was obtained by considering paths that showed a single reversal; 306 paths total, 54 with one reversal. The total number of reversals occurring in each frame is determined by ensemble summation. (B) Histogram corresponding to data shown in (A); 21% reversal. Visual tracking of individual cells indicated that, for Flx15, paths showing two or more reversals in a 5-s period were most often the result of out-of-focus cells, immotile cells, and occasional tracking errors. As a measure of response, we use the ratio of the number of paths showing one reversal to the total number showing zero to one reversal. The net tracking efficiency is 80-90%. (C) Stimulated cells. A repellent light was turned on 1 s after initiation of data collection. The bar indicates the duration of the stimulus. 974 paths total, 548 with one reversal. (D) Histogram corresponding to data shown in C; 73% reversal. Note the population shift from the column representing paths with no reversals to that for paths with one reversal.

Response latencies were determined from reversal distributions generated by considering only those paths for which the automated tracking system shows a single reversal, which correlated closely with reversals observed by visual tracking. After an ensemble summation of the number of reversals occurring at each frame, the reversal distribution is plotted as a function of time (fig. 3, and C). Delivery of light stimuli was precisely controlled by means of an electronic shutter triggered 1 second after the event marker which initiates the data collection process. Minimum latency times are reported as the first point after delivery of stimulus at which a response above background could be detected.

Histograms of the number of reversals occurring per path are shown in fig. 3 (B and D). Comparison of visual tracking of individual cells with that done by computer indicated that for strain Flx15, paths showing two or more reversals within the 5-seconds period were caused by immotile cells, out-of-focus cells, and occasional tracking errors. The program was reasonably efficient in correctly identifying actual reversals (83% of those detected visually) and paths containing no reversals (92% of those detected visually). As a result of the cell's slow translational motion and fluctuating digitized outline, the analysis occasionally gave rise to artificial reversals. The problem was minimized by smoothing the paths a number of times. The paths shown in fig. 1 have been smoothed nine times over a window of five frames.

1.2.7 Electron Microscopy

1.2.7.1 Shadow casting

A drop of cell suspension was first placed for 1 minute on a 400-mesh copper grid covered by a carbon-coated formvar film which was pre-treated by glow discharge, dried for 1 minute at room temperature, then shadowed with platinum/carbon at an angle of 25 degree and coated with a backing film of carbon using electron beam evaporating guns.

1.2.7.2 Negative staining

A drop of cell suspension was left for 1 minute on the grids as above and then sucked off with filter paper while negative stain solution (2% of phosphotungstic acid, pH 7.0 or uranyl acetate, at pH 4.8) was added from the other side for ~ 5 seconds. Excess staining solution was sucked off with the filter paper and the samples were allowed to dry. By this method most of the bacterial cells remain intact. Micrographs were recorded with either a JEOL electron microscope JEM 100-CX operating at 80 kv or a Phillips EM 300 operating at 60 kv.

1.2.8 Measurement of helical parameters of flagella

Measurements of wave length, amplitude and pitch angle were made from enlarged prints. Undistorted specimens were

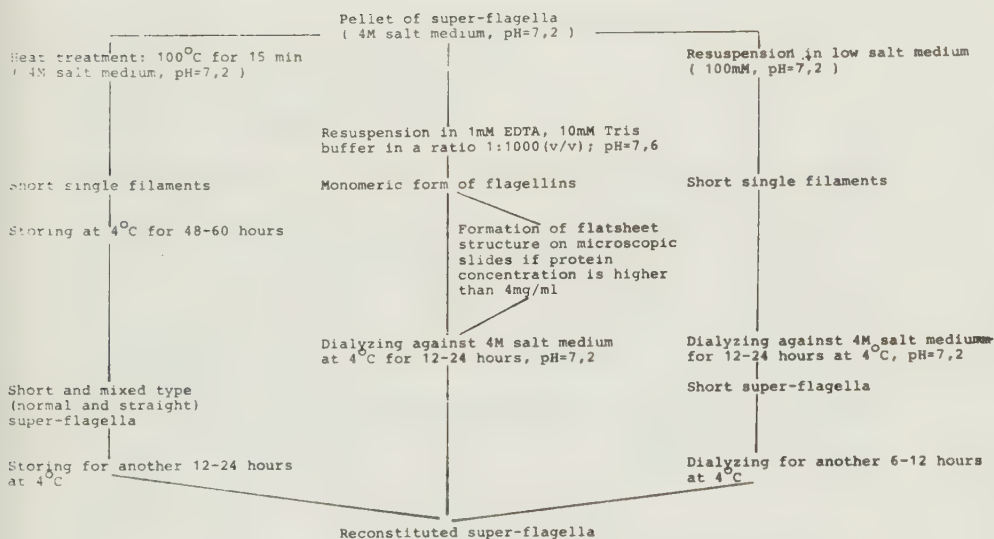
selected, and the distance between a number (5 to 10) of wavelengths was measured. For determination of amplitude, straight lines were drawn through the mean peak density on each side of the wave, and the distance between the 2 parallel lines was taken as the helical amplitude. For pitch angle, a straight edge was lined up so that it gave the best approximation to the visual average of the pitch of successive waves. Wherever possible, pitch angle was measured from left to right as well as from right to left, to reduce systematic error resulting from the illumination pattern (Macnab and Ornston, 1977).

1.2.9 Isolation and purification of flagella

The cell culture was grown to the late stationary phase (5 to 6 days) and then the cell suspension was centrifuged for 30 minute (6000 g at 10°C). The supernatant containing super-flagella was first centrifuged for 15 min (15,000 g at 15°C) separated from a pellet and centrifuged for another 2 hours (40,000 g at 4°C). The pelleted super-flagella were resuspended in basal salt medium. The flagella were further purified by a caesium chloride density-gradient centrifugation in basal salt for 50 hours ($d=1.36 \text{ g/cm}^2$; 75,000 g at 4°C). The gradient showed two white bands and the majority of flagella was found in the lower band when examined by dark-field microscopy and by SDS/polyacrylamide gel electrophoresis.

1.2.10 Reconstitution of purified flagella

Dissociation and reconstitution of flagella purified by CsCl density-gradient centrifugation was carried out as shown on the flowsheet(scheme 1).



Scheme 1:

Flowsheet for the dissociation and reconstitution procedure for super-flagella from *H. halobium* strain M-175.

1.2.11 Polymorphic transition of flagella

Reconstituted super-flagella were resuspended in basal salt medium and pH was changed by careful addition of 1 M NaOH or 1 M HCl to the suspension. A 5 μ l sample of the suspension was placed on a microscope slide, covered by a cover slip and observed by dark-field microscopy.

1.2.12 Preparation of antibodies

Purified flagella were injected subcutaneously into white male chickens with Freund's complete adjuvant. A booster injection with the same amount of purified material (final concentration 100 μ g/ml) mixed with Freund's incomplete adjuvant was injected after 2 weeks. Serum preparation was carried out 4 weeks after the first injection. The activity of the antibodies produced was analysed by the capacity of the serum to cause aggregation of the flagellated cells. The serum obtained could be used at a dilution of 1:200.

1.2.13 SDS-Polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gel electrophoresis was performed essentially by the procedure of Laemmli (1970), with modifications described by Randal and Hardy (1977).

Solutions for SDS-Polyacrylamide GelsSolution I: 30% acrylamide, 0.8% bisacrylamide

Acrylamide	300 g
N,N methylene-bisacrylamide	8 g
glass-distilled H ₂ O	to final vol. of 1000 ml

filtered through Whatman GF/C filter mounted in a Buchner funnel.

Divided into 100 ml portions in small bottles,
stored at 4°C.

Solution IIa: Lower gel buffer, 3 M Tris HCl pH 8.7

Tris-hydroxyaminomethane (high grade)	363 g
glass-distilled H ₂ O	900 ml
adjusted pH with 10 M HCl (~ 50 ml)	
glass-distilled H ₂ O	to final vol. 1000 ml

filtered as above, stored at 4°C.

Solution IIb: Upper gel buffer, 0.5 M Tris,

0.22M NaH₂PO₄ pH 7.8

Tris-hydroxyaminomethane (high grade)	30 g
glass-distilled H ₂ O	300 ml
NaH ₂ PO ₄ (1 M = 69 g/500 ml) used to adjust pH to 7.8	
glass-distilled H ₂ O	to final vol. 500 ml

7x Running Buffer: 0.2 M Tris, 1.534 M glycine

pH 7.8

Tris-hydroxyaminomethane (low grade)	114 g
glycine (medicinal)	691.2 g
glass-distilled H ₂ O	to 6000 ml

20% SDS: for gel solutions

SDS (high grade)	80 g
glass-distilled $H_2O(60^{\circ}C)$	35 g
glass-distilled H_2O	to final vol. 400 ml
filtered through Whatman GF/C	

20% SDS: for running buffer

low grade SDS, same as above, not filtered

1:1 Gel Buffer: 0.75 M Tris, 0.2% SDS,

0.1% TEMED(v/v)

sol. IIa	200 ml
20% SDS	8 ml
TEMED	0.8 ml
glass-distilled H_2O	to final vol. 800 ml

Spacer: 5% acrylamide, 0.13% bisacrylamide, 62 mM Tris,
 27.5 mM NaH_2PO_4 , 0.1% TEMED

Sol. I	83 ml
Sol. IIB	62.5 ml
20% SDS	2.5 ml
TEMED	0.5 ml
glass-distilled H_2O	to final vol. 500 ml

Running Buffer: 28 mM Tris, 220 mM glycine,

0.1% SDS

7x running buffer	1000 ml
20 SDS%	35 ml
glass-distilled H_2O	to final vol. 7 liters

10x Sample Buffer: used to make 5x S.B.

20% SDS	10 ml
Sol. IIb	8 ml
β -mercaptoethanol	2 ml
Bromphenol blue	5 mg

5x Sample Buffer: to yield as 1x: 20 mM Tris,

8 mM NaH_2PO_4 (pH 7.8), 1% SDS, 1% β -ME	
10x S.B.	10 ml
glycerol	10 ml

10% Amonium persulfate Solution:

APS	10 g
glass-distilled H_2O	to 100 ml
Distributed in 1 ml aliquots and frozen. A new aliquot thawed for each day's gels.	

Stain I

Coomassie Brilliant Blue	2 g
distilled H_2O	3.25 liters
isopropanol	1.25 liters
acetic acid	0.5 liters

Destain

acetic acid	0.5 liters
distilled H_2O	4.5 liters

For 0.5 mm Gel: all volumes are in ml

	10%	14%	15%
H ₂ O	4.2	0.8	0.0
1:1 Buffer	12.5	12.5	12.5
Sol. I	8.3	11.7	12.5
APS	0.18	0.18	0.18

1 mm Gel:

H ₂ O	5.0	1.0	0.0
1:1 Buffer	15.0	15.0	15.0
Sol. I	10.0	14.0	15.0
APS	0.21	0.21	0.21

Low bis Gel Solution:

Acrylamide(high grade) 30 g
 Bisacrylamide 0.2 g
 glass-distilled H₂O to a final vol. 100 ml
 filtered through Whatman GF/C

11% Low-bis gel

H₂O 4
 1:1 Buffer 15
 low-bis Sol 11
 adjusted pH to 8.2
 APS 0.21

2.2.14 Labeling of cells with isotopes and preparation of total protein

Cells were grown in complex medium to a density of approximately 2.5×10^8 cells per ml, harvested by

centrifugation, washed three times in basal salt medium with 0.1% arginine, and suspended at the same density and in the same buffer containing 30 to 100 μ g of puromycin per ml. After 10 min L-(methyl-³H-methionine) was added to a final concentration of 2 to 4 μ M. Methylation was stopped at various time depending on the specific experiment by mixing 100 μ l of the labeled cell suspension into ice cold acetone (1:9, v/v). Proteins were collected by centrifugation, washed in cold acetone(50%) and dried for 10 minutes in a Speedvac before being dissolved in electrophoresis sample buffer. The samples were boiled for 3 to 5 minutes prior application to the gels.

2.2.15 Quantitation of methyl ester radioactivity

Volatile/base-labile radioactivity was analyzed after the gels were stained, destained and dried. The gel was then sliced with razor blade slicer(2 mm pieces, 1 cm wide). Each slice was placed in a 0.5-ml microfuge tube which was then set inside of a 15-ml scintillation vial containing 6 ml of Scintillation fluid. Sodium hydroxide (0.1 ml of a 1N solution) was added to each microfuge tube and the scintillation vials were stoppered. Vials remained at room temperature for 24 hours. Care were taken at all times to avoid bumping or tipping the vials as this may introduce scintillation fluid into the microfuge tube containing the gel slice (Chelsky et al,1980). For each samples in the scintillation counter 5 minutes was enough for total counting.

2.2.16 Measurement of radioactive methanol production by continuous-flow assay

Volatile radioactive methanol in cell supernatant fractions was measured by the method of Chelsky (1980). Vapor phase transfer of radioactivity was performed in scintillation vials at room temperature for 24 hours (Kehry et al, 1984). The filter assembly (Gelman acrodisc, 0.2 μ m) used in all experiments was connected at the outlet end to a fraction collector. The inlet (luer fitting) was adapted to peristaltic-pump tubing (0.42 ml/min or 1.5 ml/min, Evergreen Scientific). The filter was washed with 10 ml of basal salt medium before the addition of cells. Cells were labeled for 40 min, harvested by centrifugation and washed three times with wash medium. Then the cell suspension containing between 1.5×10^9 and 2.5×10^9 cells were transferred onto the filter by pumping and maintaining a continuous flow of chase medium (basal salt plus 0.1% arginine medium with methionine) at room temperature. Fractions of the chase medium passed through the filter were collected from the time on the cells were loaded. The fraction time was 1 min or 18 sec. Addition of attractant or repellent was provided by switching the inlet tube to desired solution, which was prepared before in chase medium. At various times photostimuli were given by switching on and off a projector having appropriate light filters.

C. RESULTS

3.1 Flagellar apparatus of Halo- and Square bacteria

The problems of photographic documentation of bacterial flagella in dark-field microscopy have been discussed in detail by Macnab (Macnab, 1976). Using his guidelines, the optimal conditions for photographing halo- and square bacterial flagella in 4 M-basal salt medium was determined. As mentioned by Macnab, the choice of objective was critical for optimum image quality. Considerable experimentation indicated that 50x dry fluorite objective gives a good combination of image brightness, contrast, and freedom from flare. For the best video recordings, however a 100x oil immersion objective with numerical aperture (NA) 1.32 to 0.6 was used because of low scattering power of flagella and considerably high scattering of 4 M-salt medium.

3.1.1 Halobacteria

3.1.1.1 Dark-field microscopy

Flagella of *H. halobium* cells were found at one (Fig. 4A) or both (Fig. 4B) poles. When the ratio of monopolarly to bipolarly flagellated cells was followed during growth, it was found that in the logarithmic phase, the percentage of monopolarly flagellated cells was high, but in the

stationary phase, cells were predominantly bipolarly flagellated. Sometimes long filamentous cells were observed with flagellar bundles arising at random points along the cell body (Fig. 4F). Thus, during growth, division of flagellated cells occurs in two ways: (1) a bipolarly flagellated cell divides into two monopolarly flagellated daughter cells (Fig. 4D); (2) a monopolarly flagellated cell divide into a monopolar and a non-flagellated cells (Fig. 4E). Figures 4D1 and E1 show the septa of two dividing cells.

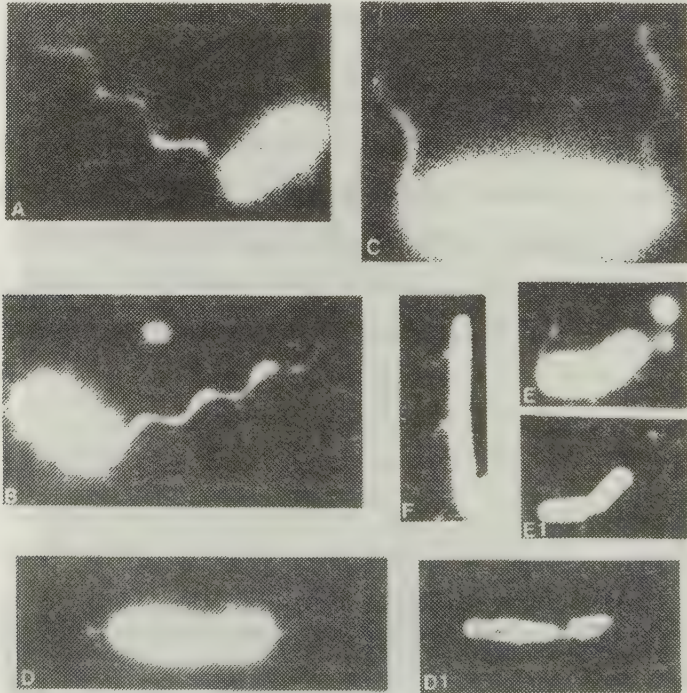


Figure 4:

Dark-field micrographs of *H. halobium* cells that are: A and B, monopolarly, 19,500x; C, bipolarly, 19400x; and F, lophotrichously, 2500x, flagellated. The lower panel shows the division of: D, bipolarly, 2500x; E, monopolarly, 2500x, flagellated. D1 and E1 are prints of short-exposure films that show clearly the dividing cell bodies. Micrograph F was taken from the video film.

3.1.1.2 *Halobacterial flagella have right-handed helical form*

It is well-established that most eubacterial flagella form a left-handed helix at pH 7(Asakura et al., 1966); in contrast, the complex flagella of soil eubacteria like *Rhizobium meliloti* 2011 and *Rhizobium lupini* H 13-3 form a right-handed helix (Götz, 1982). We determined the helical handedness of halobacterial flagella using the optical method reported by Shimida et al. (1975). The method is best understood by considering a bundle of left-handed helical filaments, lying on the surface of a slide glass(Fig. 5). If parts of the bundle near planes a, b and c are illuminated successively and focused by raising the specimen stage discontinuously, the images of types x, y and z will be obtained in succession. If a bundle of right-handed helical filaments is examined, images of types z, y and x will be observed, corresponding to planes a, b and c respectively. Figure 6 presents a series of photographs focusing on (a) the top, (b) the middle and (c) the bottom of a halobacterial flagellar bundle and thereby clearly demonstrates that halobacterial flagella have a right-handed helix.

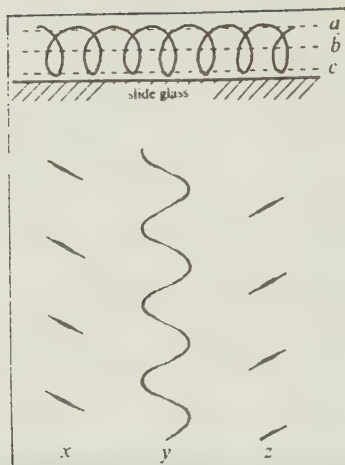


Figure 5:

The optical method used for the determination of helical handedness(Shimada et al).

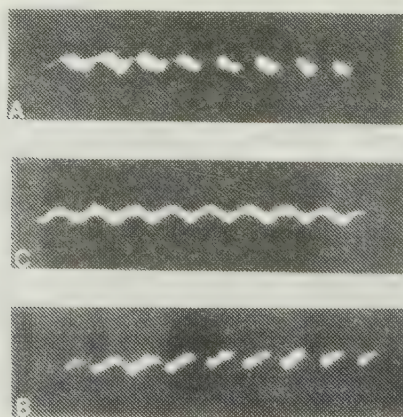


Figure 6:

A set of micrographs focusing on (a) the top, (b) the middle and (c) the bottom of a flagellar bundle, 7600x, at pH 7.0.

3.1.1.3 CCW(counter-clockwise) and CW(clockwise) rotation of flagella

Looking along flagella towards the eubacterial cell body, CCW rotation of left-handed helical flagella exerts a pushing force on the cell and a wave travelling outward from the cell was defined as propagating positively (Macnab and Ornston, 1977). Using dark-field microscopy, we video-recorded swimming and reversing of single halobacterial cells and could visualize clearly the rotating flagella(Fig. 7). As expected for a right-handed helix, CW rotation of the flagellar bundle produced a pushing force on the monopolarly flagellated cell(scheme 2). Anti-cooperative rotation, that is, CW and CCW rotation of the two flagellar bundles causes a bipolar cell to swim from left to right or right to left. There are nine possibilities: each stop, CCW and CW mode have three possibilities (Scheme 2A). Sketches of principal modes of motility observed in *H.halobium* are given in scheme 2 B.

Left-handed helical flagella in *Salmonella* undergo phase transition due to the torsion generated when rotatory motor is operating reverse(i.e CW sense) and the flagella tend to become right-handed. In halobacteria the phase transition is also visible during the change from CW to CCW rotation of flagellar bundle, but the helicity remain the same (Fig. 8).

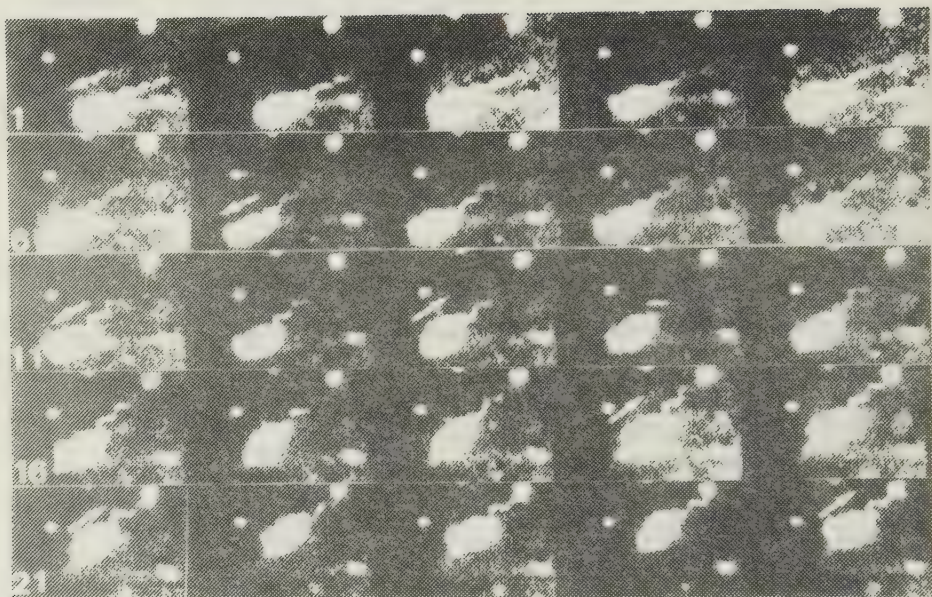


Figure 7:

Dark-field microphotographs of swimming and reversing of a monopolar halobacterial cell (2500x). The arrows indicate the direction of translational movement. Micrographs were taken from the video film at intervals of 0.4 s.

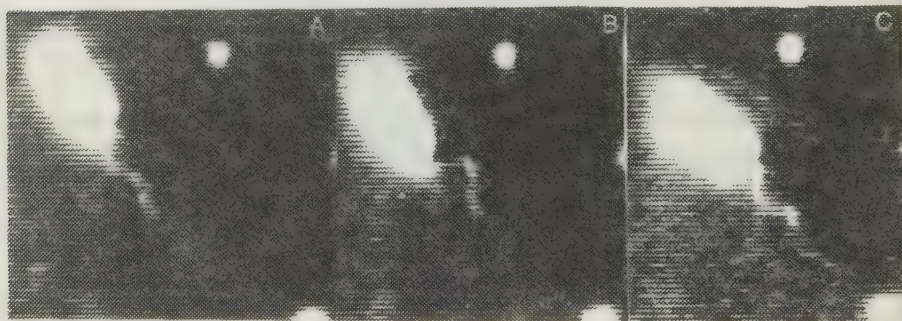


Figure 8

Phase transition of flagellar bundle during the change of rotation from CW (A) to CCW (C) through Stop (B) of monopolarly flagellated *Halobacterium halobium*.

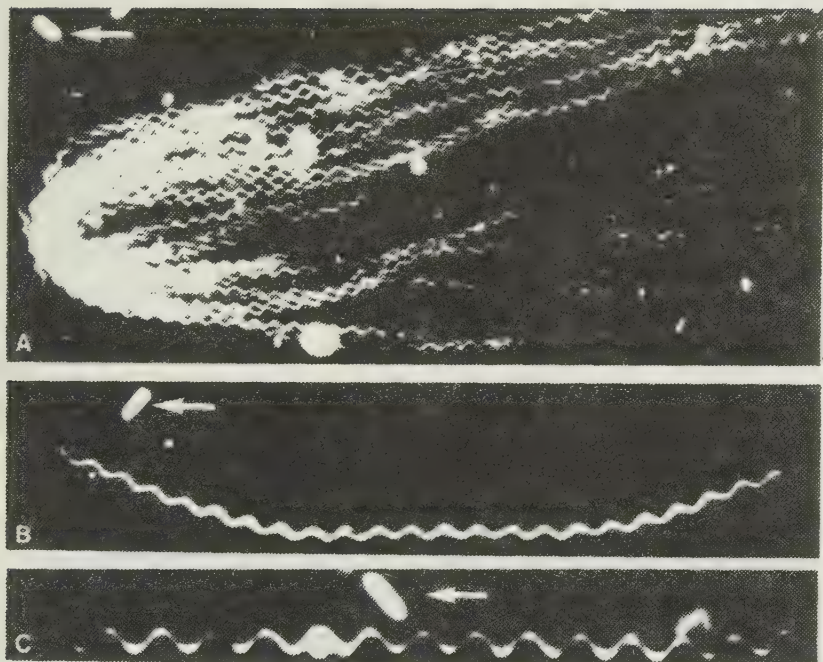
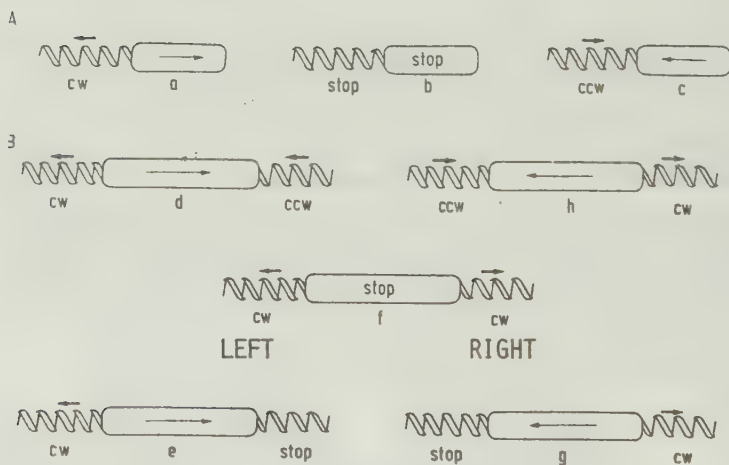


Figure 9

Dark-field micrographs of super-flagella of *H. halobium*. The arrows point to a single halobacterial cell body. (c) 11,500x and (b) 9700x are single super-flagella of *H. halobium* M-175 and L-07.

RIGHT LEFT	STOP	CW	CCW
STOP	STOP	RUN	RUN
CW	RUN	STOP	RUN
CCW	RUN	RUN	STOP

A:



Scheme 2

B:

Scheme 2

A: Different probabilities of Stop, CCW and CW mode of flagellar rotation. B: Sketches of principal modes of motility observed in *Halobacterium*. A: Monopolar cells; B: Bipolar cells show five possible combination of flagellar rotation (out of nine).

3.1.1.4 Super-flagellar phenomenon

Dundas(Dundas, 1977) reported that only stationary phase cultures contain large numbers of loose flagella, which aggregate into thick spiral bundles. I also observed the production of these bundles (Fig. 9A, B and C), but in the strains I used they could be observed at all phases of growth. They were designated as super-flagella. Generally, these super-flagella were 10 to 20 times longer than the cell body(Fig. 9B and C). Electron microscopy of super-flagella showed that each consisted of more than 200 individual filaments (Fig. 10A and B).

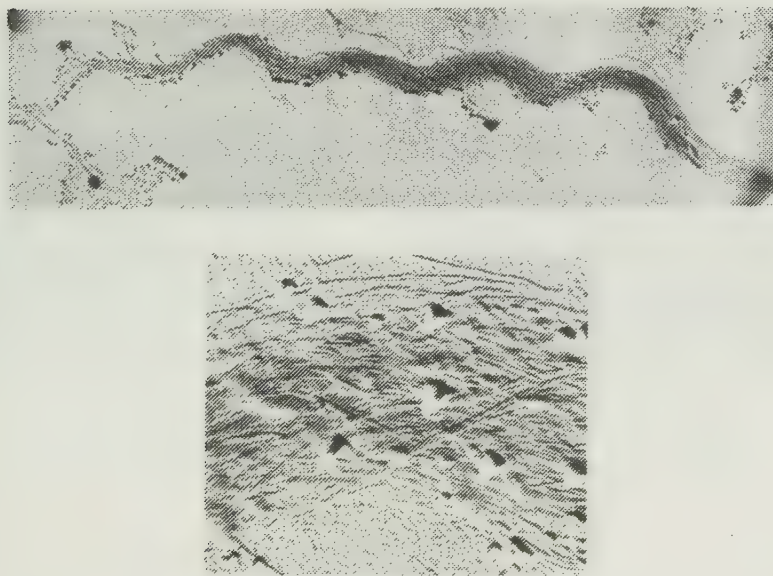


Figure 10

Electron micrographs of super-flagella of *H. halobium*. (B) Lower magnification 9800x; (A) part of (B) at higher magnification (70,000x).

3.1.1.5 Flagellar bundles never dissociate during the change of direction of rotation from CW to CCW or vice versa

Dark-field micrographs can not clearly distinguish the single filaments from bundle formations because of low scattering light of flagella in 4 M-salt medium. According to Houwink(Houwink,1956), halobacteria have five to ten filaments only at one pole. In eubacteria (peritrichous form), CCW rotation of the LH flagella causes a bundle to form and the cell runs. CW rotation causes the flagellar bundle to dissociate (Macnab and Ornston, 1977). Video pictures and observation by eye of moving and stationary halobacterial cells demonstrated that flagellar bundles never fly apart during the change of the direction of rotation(Fig. 7 and Fig. 8). Thus to demonstrate that in dark-field microscopy observed flagellar bundle consists of several filaments, I used electron microscopy. Figures 11 and 12 show that halobacterial flagellar bundles consist of several filaments at one or both poles.

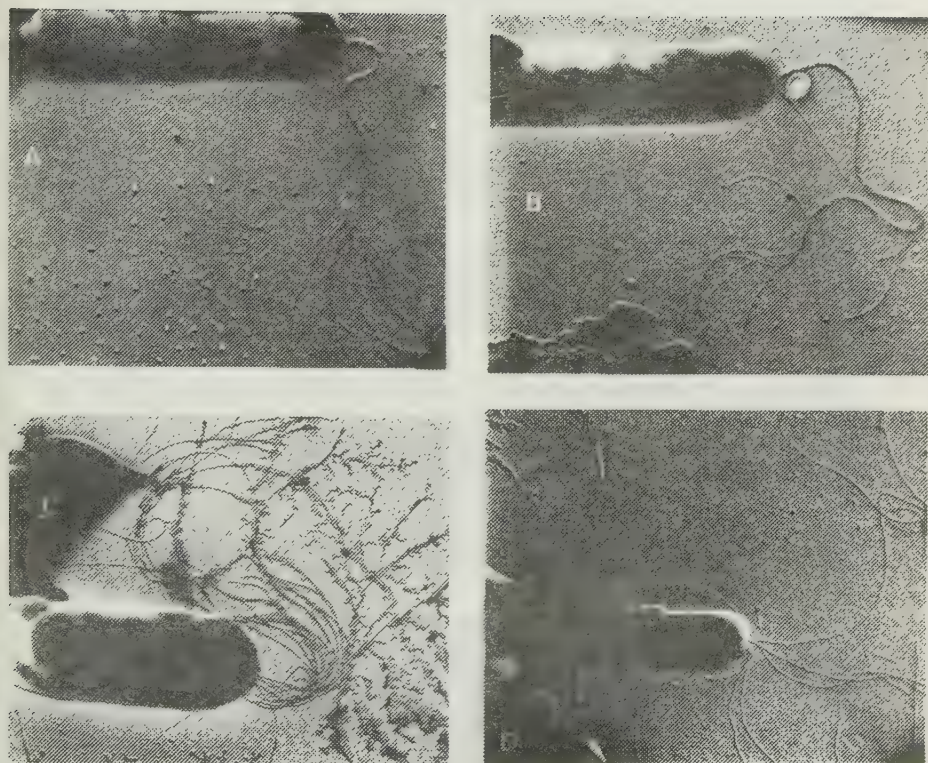


Figure 11

Electron micrographs of monopolarly flagellated H. halobium. (A), (B) show tightly coupled bundle; (C) and (D) show that the bundles were dissociated to several single filaments.

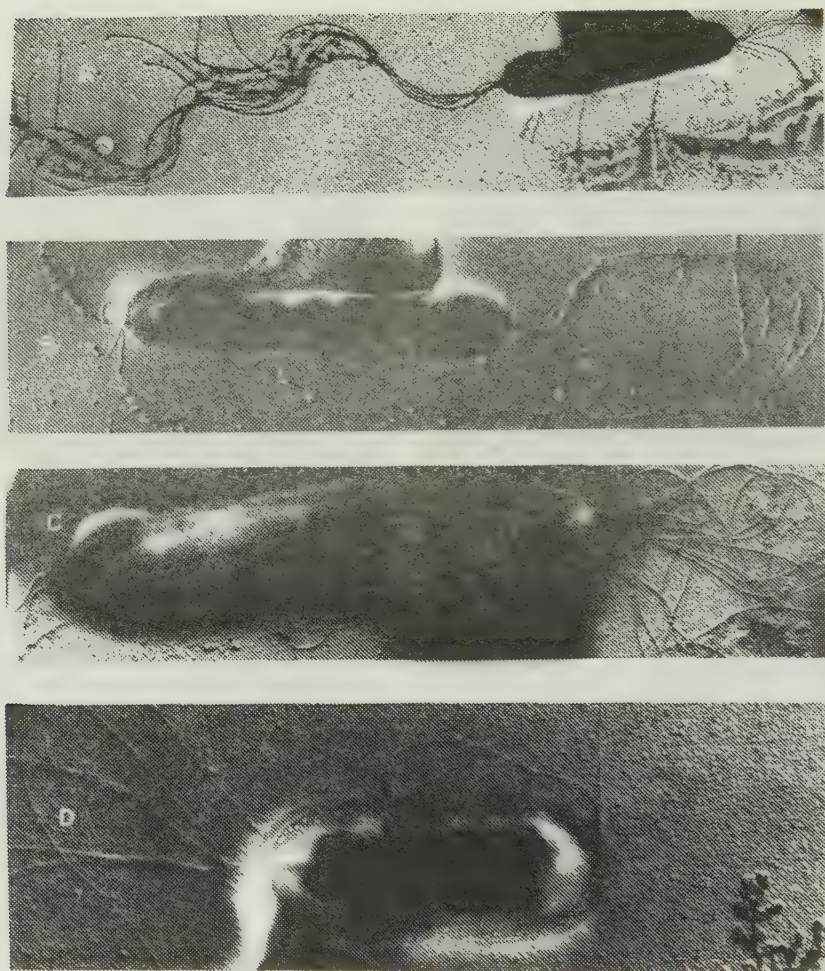


Figure 12

Electron micrographs of bipolarly flagellated H. halobium.

3.1.2 Square bacteria

The above results show that halobacterial flagella are distinctly different from eubacterial flagella. To see if this difference may be common among halophilic archaeobacterial species, we examined the flagella of Square bacteria which are also a member of archeobacteria and have the same ecological niche as halobacteria.

3.1.2.1 Different types of flagellar attachment to the cell body

Dark-field micrographs of cell suspensions show large flagellar bundles resembling the super-flagella of rod-shaped halobacteria. The flagellar bundles (Fig. 13, 15) appear in the light microscope detached from the cell body, or attached as the super-flagella of halobacteria. Microscopic observation of swimming cells reveals that flagellar bundles, seemingly detached from the cell body, nevertheless rotate in coordination with movement of the cell body. The explanation of this behaviour is given by the pictures of Figure 14-16, which summarize three apparent types of association of flagellar bundles with a cell body. Figure 15A is taken from video film that recorded a swimming square bacterium. The flagellar bundle seen in the light microscope forms well behind the cell body from smaller bundles or single filaments not visible in the light microscope arising at different points on the cell surface. Electron microscopy clearly distinguishes the separate

filaments(Figure 14). A second type of flagellation is shown in figure 15B where the flagellar bundle arises directly from the cell body. An electron micrograph with this type of flagellar arrangement is shown in Figure 14E and Figure 16A. The third type of flagellation consists of several bundles (Fig. 15C and D). The corresponding electron micrographs are shown in Figures 14D and F. Swimming square bacteria do not always carry flagellar structures visible in the dark-field microscope. The explanation is given by the examples in figures 14A-C showing single filaments that are mono- or peritrichously attached to the cell body. The electron micrographs of Figure 14 do not allow a conclusion about the points of attachment of the flagella; they may be attached either to the flat surface of the cell or to the edges. For example in Figure 14D and F the flagellar structure can be seen arising from the flat surface; in other cases (Fig. 14A and B) the flagella seem to end at the edges though they may be attached to the flat surface on the other side of the specimen.

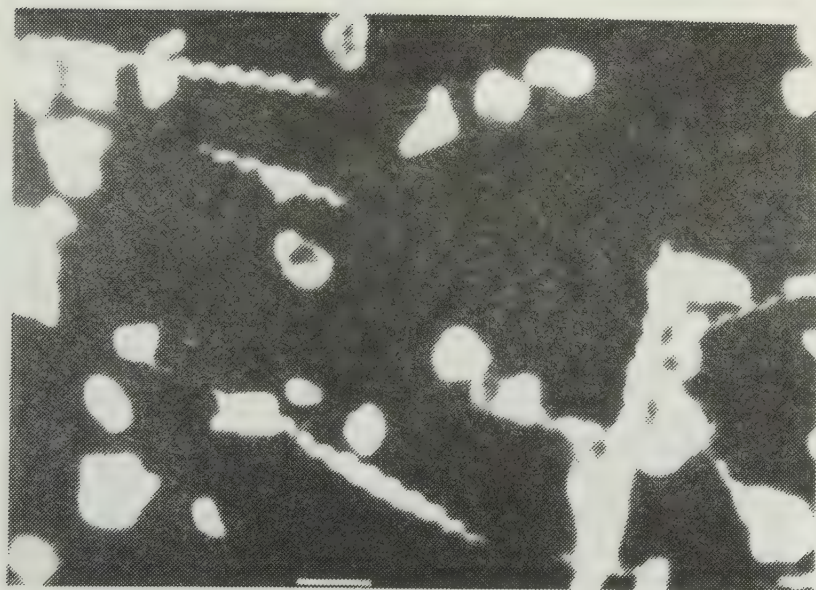


Figure 13

Dark-field micrograph of a square bacterial culture. Detached and cell body-attached flagellar bundles occur but can not always be distinguished (see also Figure 15A).

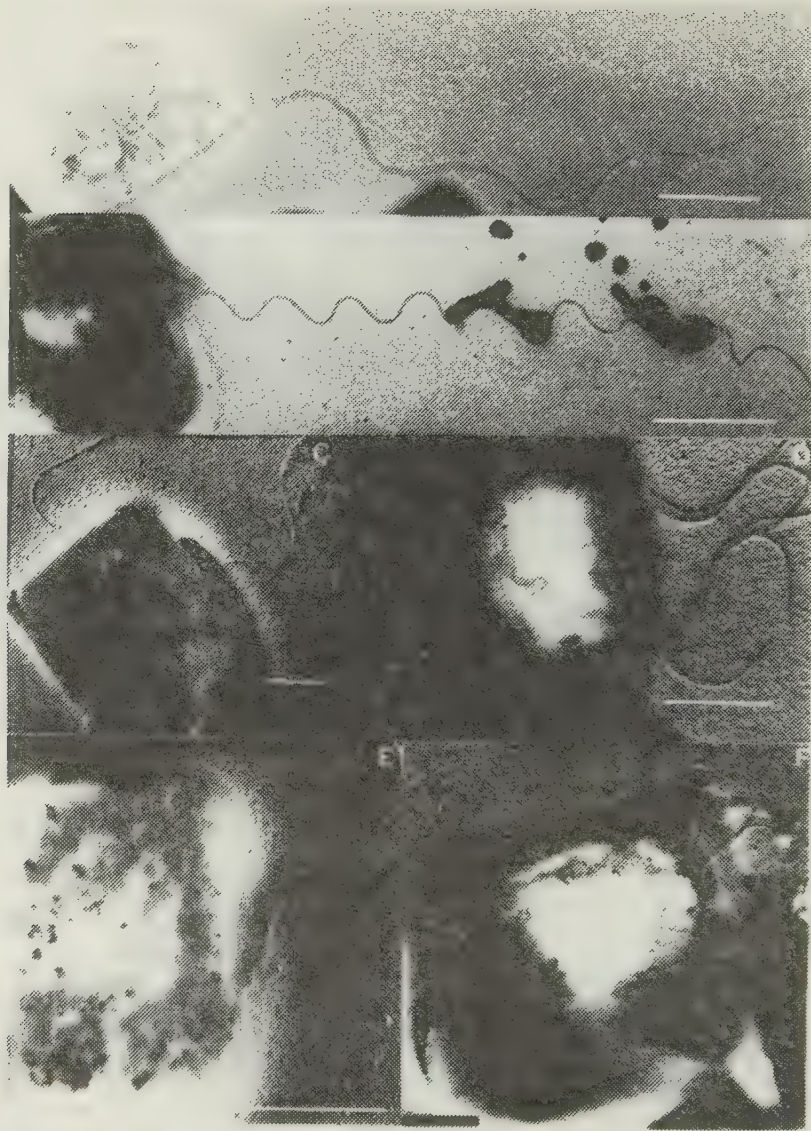


Figure 14

Electron micrographs of flagellated square bacteria. (A) and (B) Monotrichously flagellated cell with a single flagellum negatively stained with phosphotungstic acid (A) and uranyl acetate (B). (C) Peritrichously flagellated cell (two individual flagella). (E) Cell body with a single giant flagellar bundle. (D) and (F) Peritrichously flagellated cell with two flagellar bundles. The bars indicate 0.8 μm .

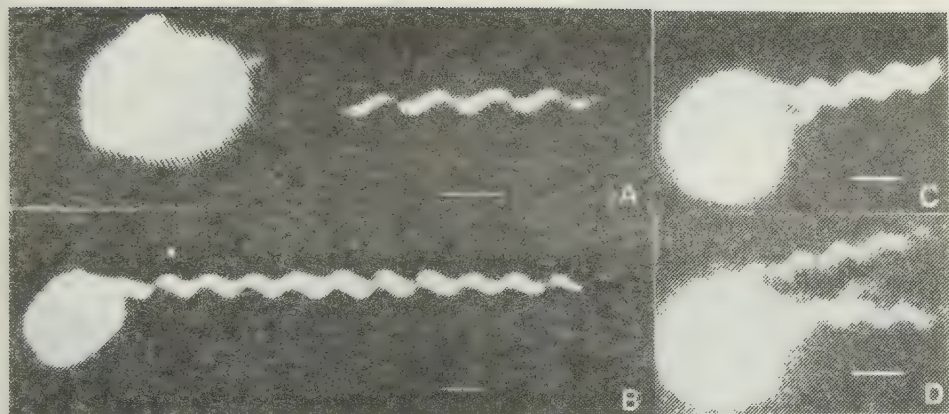


Figure 15

Dark-field micrographs of square bacteria. Various examples of flagellation are shown. (A) Taken from video film: flagellar bundle formed from individual filaments extending from different locations on the cell surface. (B) Flagellar bundle arising from one location on the cell surface. (C) and (D) Several bundles arising at different locations on the cell surface. The bars indicate 1 μ m.

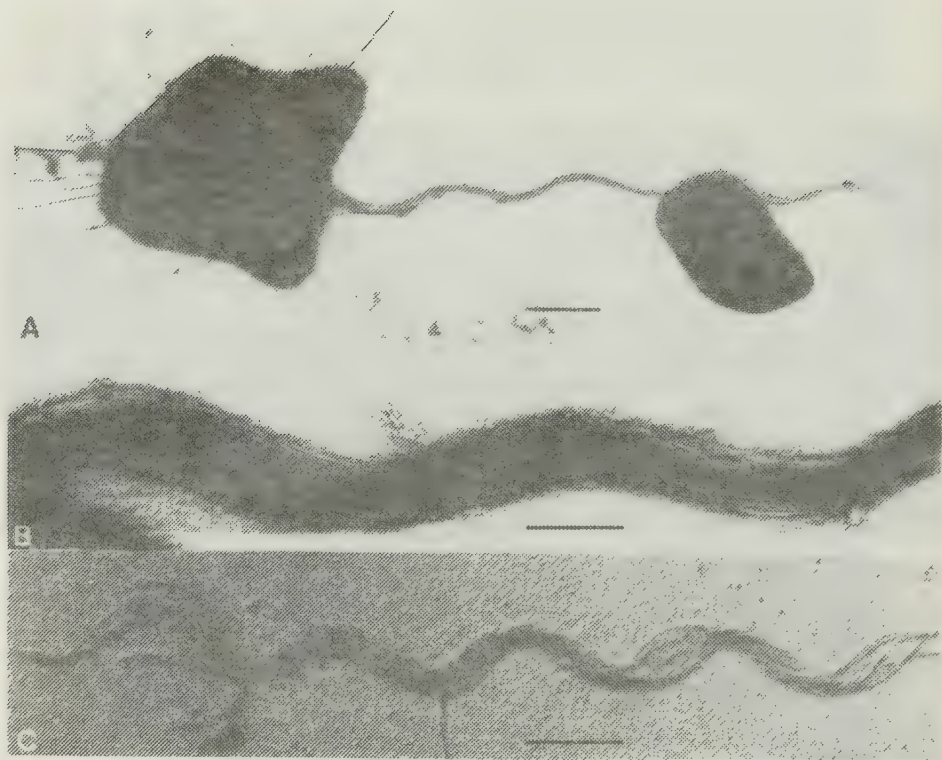


Figure 16

A: Electron micrograph of a negatively stained (1% uranyl acetate at pH 6.8) square bacterial cell having a giant flagellar bundle. **B:** Detached flagellar bundle of several filaments at higher magnification (negatively stained). **C:** Detached flagellar bundle after shadowing with platinum/carbon. The bars indicate 1 μm .

3.1.2.2 Square bacterial flagella have also right-handed helical form

As for halobacterial flagella, the helical handedness of square bacterial flagellar bundles was also determined and found that they also have a right-handed helical form. Figure 17 presents a series of micrographs focusing on the top (a); the middle (b) and the bottom (c) of a flagellar bundle and thereby clearly demonstrates that square bacterial flagella have a right-handed helical form.

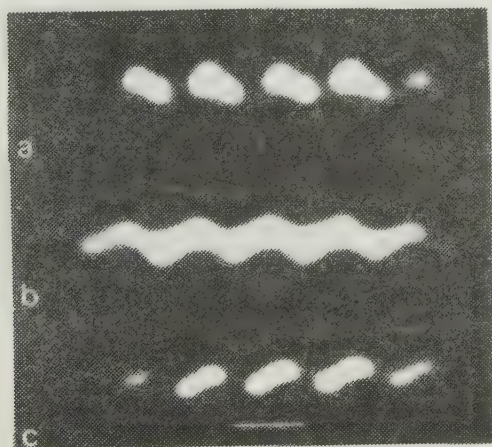


Figure 17

A set of micrographs focusing on the top (a), the middle (b) and the bottom (c) of a flagellar bundle of square bacteria at pH 7. Bar equals 1 μ m.

3.1.2.3 Square bacterial flagellar bundles also do not dissociate during the change of direction of rotation

Swimming and reversing of square bacterial cells were video recorded by dark-field microscopy (Macnab, 1976) and clearly showed rotating flagella (Fig. 18). As expected for a right-handed helix, CW rotation of the flagellar bundle produces a pushing force on the square bacterial cell (Fig. 18 frame 1-15). Thus, the motility pattern for flagellated square bacteria consists of smooth swimming (runs) in a forward direction, stops, and then runs in the opposite or in the same direction. Video pictures and direct observation of swimming and stationary square bacterial cells with rotating flagellar bundles demonstrated that the bundles never fly apart during the change of direction of rotation from CW to CCW or *vice versa*. Thus as in halobacteria, the filaments of square bacterial flagella show strong lateral affinity. During the translational movement, the cell body also rotates but opposite to the direction of the flagellar rotation.

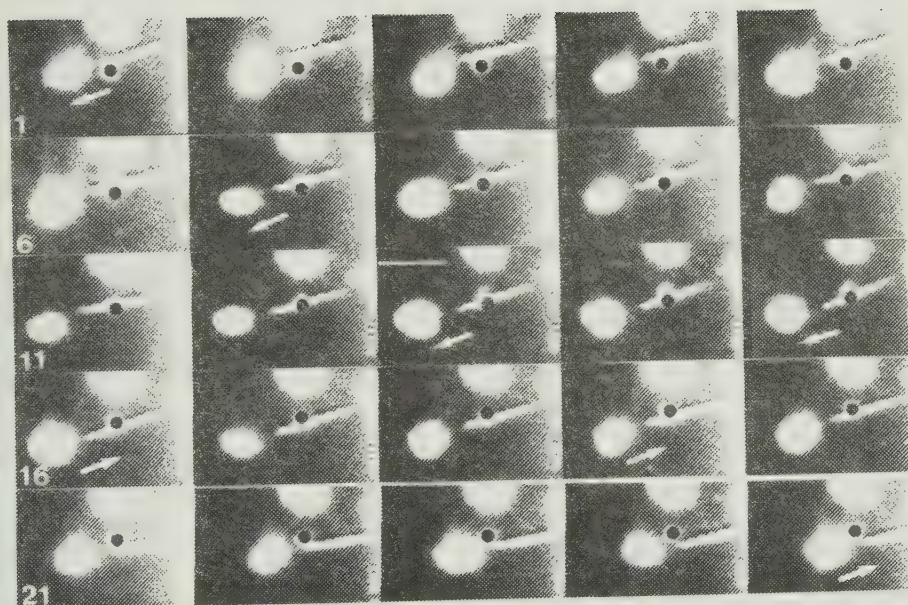


Figure 18

Dark-field micrographs of swimming and reversing of a square bacterium cell. The arrow indicates the direction of translational cell body movement. The black dot serves as a point of reference. Micrographs were taken from video film at an interval of 0.3 sec. The bar (center) indicate 4.5 μm .

3.2 Isolation and purification of flagella

3.2.1 Halobacterial flagella

Attempts to isolate halobacterial flagella by traditional methods resulted in excessive contamination of the preparations by cell debris (Dundas, 1977). Therefore the phenomenon of aggregation of loose flagella into super-flagella was utilized. We chose a mutant, *H. halobium* M-175, that is an overproducer of super-flagella. Furthermore, aggregation of loose flagella into super-flagella could be drastically increased if a stationary phase culture was kept at room temperature without shaking for 24-36 hours.

Pelleted super-flagella (40,000 g, 2 h, 4°C) contained a small amount of cell debris (Fig. 19A) and SDS-PAGE confirmed this contamination (Fig. 20A). Several washings followed by centrifugation at low speed (15,000 g, 30 min) and recentrifugation of the supernatant (40,000 g, 2 h) did not reduce the amount of cell debris found within the super-flagellar pellet. In addition, the purity of the preparations was not reproducible. Thus, CsCl density gradient centrifugation was used for further purification. After 50 hours of centrifugation two bands were observed on the gradients. The majority of purified flagella was found in the lower, white translucent band when examined by dark-field microscopy (Fig. 19A) and by SDS-PAGE (Fig. 20B). The upper band contained vesicle-like particles and a small

amount of flagella.

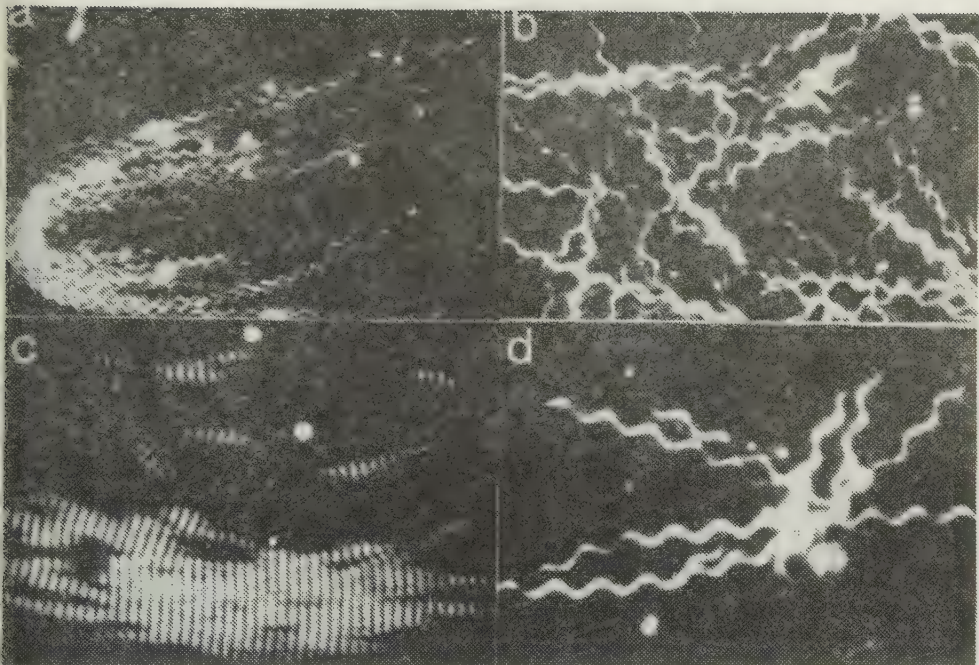


Figure 19

A set of dark-field micrographs of super-flagella from *Halobacterium halobium* strain M-175. A) partially purified (after centrifugation of 40,000x g for 2 hours, 2500x fold magnification;; b) flagella purified by CsCl gradient centrifugation 4700x; c) flat-sheet structure, 4700x; d) reconstituted flagella, 6200x.

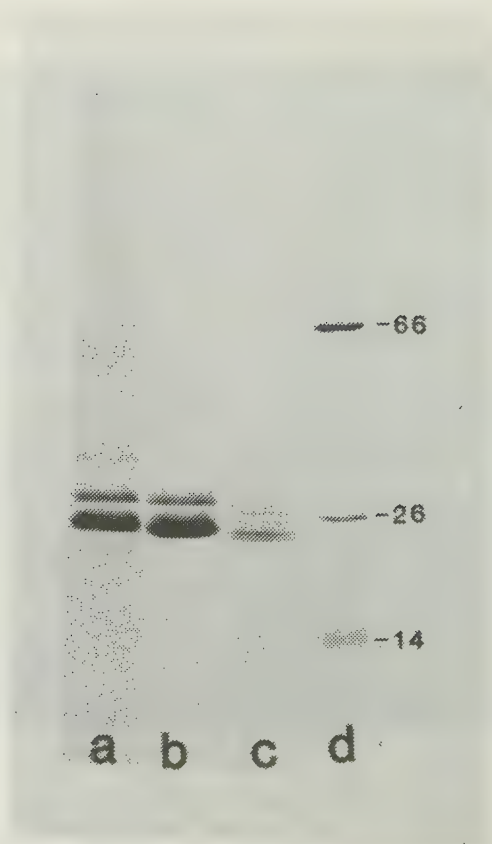


Figure 20

Analytical SDS-PAGE of different flagellar preparations from *H. halobium* strain M-175. Lanes a, b and c correspond to the micrographs a, b and d in figure 19. Lane d: marker proteins with their corresponding molecular weights indicated on the right.

3.2.2 Square bacterial flagella

Isolation and purification of square bacterial flagella were straight forward. Stationary state cultures of strain 801030/1 were freed of cells by low-speed centrifugation (30 min, 10°C at 6000 x g). The supernatant was again centrifuged for 2 hours (40000 x g, 4°C). The harvested pellet was washed with basal salt and recentrifuged as above. The purity was checked by dark-field microscopy (Fig. 21B) and by SDS-polyacrylamide gel electrophoresis. Figure 21 shows that square bacterial flagellin has a rather high molecular weight of about 73,000 and consists of only one protein compared to three proteins in *Halobacteria*. No contamination problem by cell debris during the isolation of square bacterial flagella was encountered.

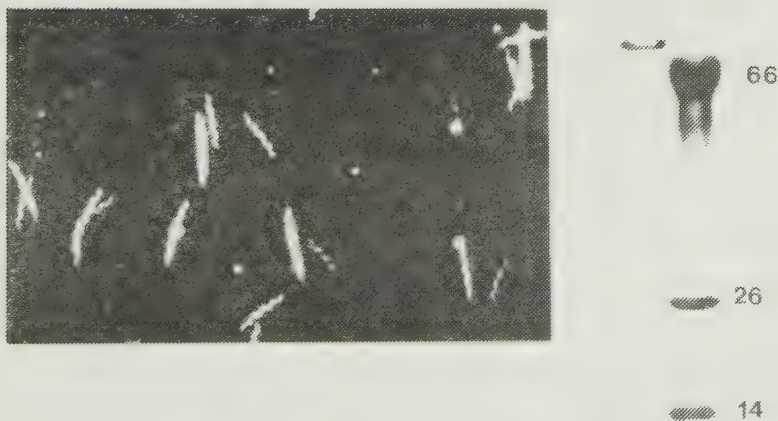


Figure 21

Purified square bacterial flagella. A) Dark-field micrograph of purified flagella; B) Lane 1: Analytical SDS-PAGE of purified flagella; Lane 2: Marker proteins with their corresponding molecular weights indicated on the right.

3.3 Reconstitution of halobacterial flagella

Pure flagella in 4 M salt suspension were treated with 1 mM EDTA, 10 mM Tris buffer (pH 7.6) in a ratio of 1:1000 (v/v). No flagellar structures could then be detected any longer by dark-field and electron microscopy. Therefore, we concluded that the flagella dissociated into their molecular components. If the concentration of the flagellins was more than 4 mg/ml a drop of this suspension placed on a microscopic slide resulted in the formation of structures shown in figure 19C. Flat sheet structures, as revealed by the single focal plane, are seen instead of coiled structures. At right angles to the longer axis dark lines indicate a waved surface or cross-section of these structures. At lower flagellin concentrations this phenomenon could not be observed and upon addition of 4 M salt to the material on a microscopic slide the typical super-flagella structures were formed within seconds. I did not succeed in analyzing the flat sheet structure by electron microscopy because during preparation of specimens the transition to the coiled super-flagellar structures occurred. Reconstitution of super-flagella was also obtained when a solution of flagellins at lower concentration was dialysed against 4 M basal salt for 12-24 hours at 4°C (Fig. 19 D). Analysis of these reconstituted super-flagella by SDS-PAGE revealed the same three bands of protein found in the starting material (Fig. 20C). This result demonstrates that all three bands contain proteins intrinsic to the flagellar structure. We designated them as flagellins Fla I,

Fla II and Fla III. The proteins retain upon reconstitution of the flagella their multiple band appearance. This heterogeneity is due to the fact that these flagellins are sulfated glycoproteins of a varying degree of glycosylation (Wieland et al., 1986).

Super-flagella dissociate into single filaments when the salt concentration of the suspending medium (Fig. 23A) was decreased to 0.8 M (Fig. 23B). A further decrease to 0.1 M (Fig. 23C) did not cause any significant change of the normal sinusoidal form. Heat treatment (100°C for 15 min) in 4 M salt also dissociated super-flagella into single filaments, but after cooling at 4°C produced mixed-type straight and normal flagella (Fig. 22A). Dissociation and reconstitution procedures are summarised in scheme 1.

3.4 Polymorphic transition of halobacterial flagella

Eubacterial flagella show polymorphic transition when exposed to different pH, viscosity or by incorporation of p-fluoro-phenylalanine (Macnab and Ornston, 1977). Halobacterial flagella in 4 M salt underwent a normal to straight transition when the pH was decreased to 2.4 (Fig. 22B); a normal to curly transition occurred if the pH was increased to 9.2 - 9.5 (Figs. 22C and 22E). Similar results were also obtained with dissociated single filaments. The decrease of salt concentration from 4 M to 0.8 M caused dissociation into single filaments (Figs. 23A and 23B). Decreasing the pH to 2.4 induced straight forms (Fig. 23D)

and increasing the pH to 10.5 caused a tendency to bending and ring formation (Fig. 23E).

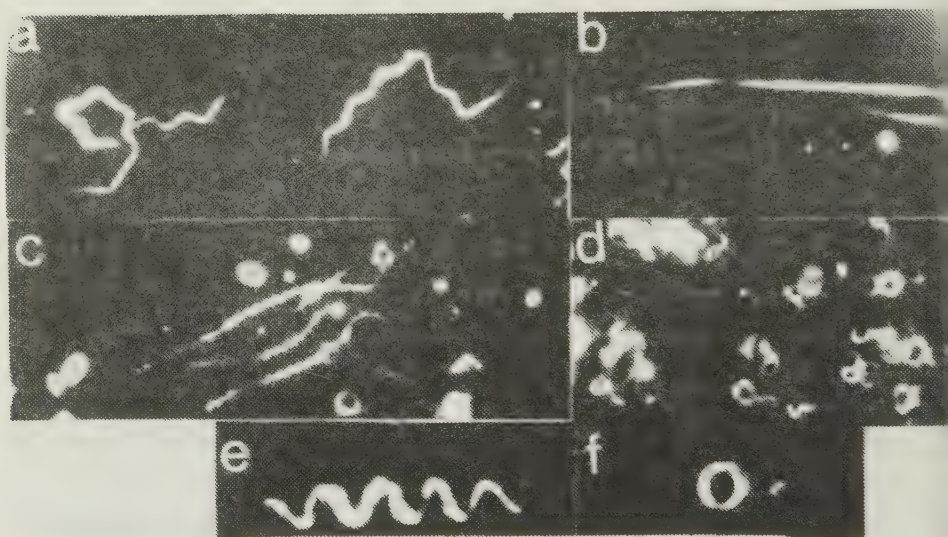


Figure 22:

Dark-field micrographs of different polymorphic states of reconstituted halobacterial flagella. a) Transition from normal to mixed (straight and normal) types induced by heat treatment, 4700 x; b) straight form at pH 2.4, 4700 x, c) curly form at pH 9.2 - 9.5 4700 x, d) ring form at pH 10.5, 4700 x, e) and f) are enlarged micrographs (26,000 and 9,500 x) of c) and d).

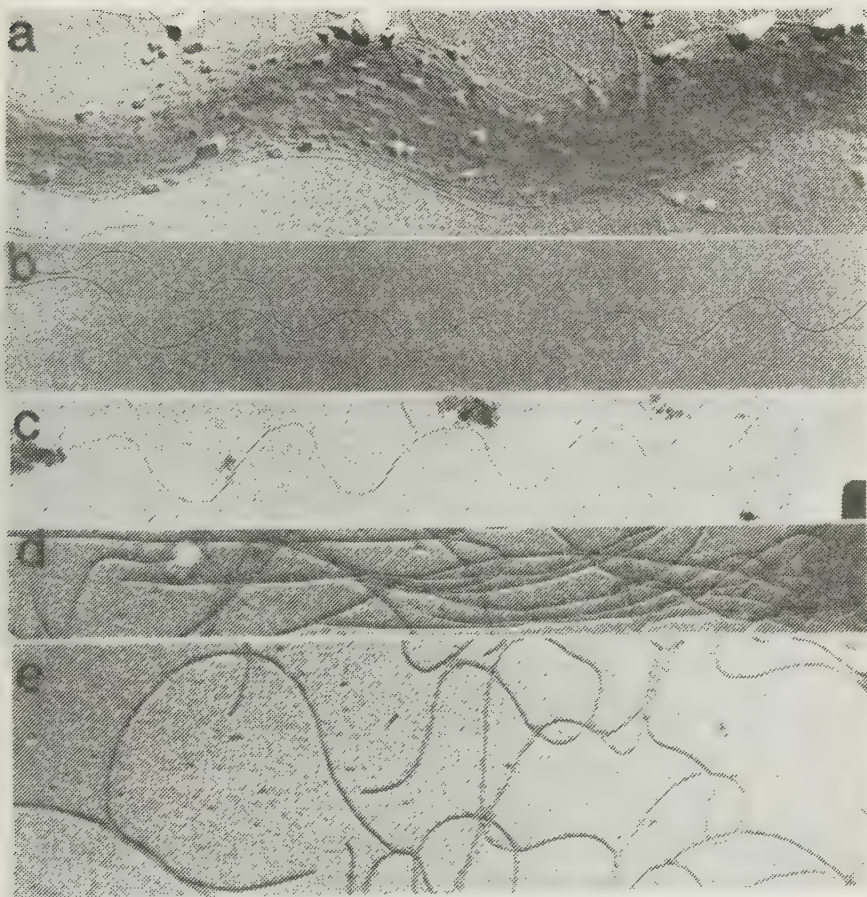


Figure 23:

Electron micrographs of different halobacterial flagellar preparations. (a) Superflagella in 4 M salt; (b) single filament in 0.8 M-salt; (c) single filament in 0.1M-salt; (d) straight form in 0.8 M-salt at pH 2.4; (e) ring form in 0.8 M-salt at pH 10.5.

3.4.1 Helical parameters and size of attached flagella, super-flagella and different flagellar polymorphs

According to the method of Hotani (1976), I determined the helical parameters of attached, super- and polymorphic flagella of *Halobacterium halobium*. Table 1 and 2 summarize the helical parameters. The length of attached flagella is close to 4 μm and increases upon super-flagella formation up to 30 μm . In eubacteria different polymorphic forms have different helical handedness (Hotani, 1976; Kamiya & Asakura, 1976). Halobacterial flagella did not change their right-handedness throughout all the polymorphic transitions.

TABLE 1

Helical parameters of attached and super-flagella of different strains of *Halobacterium halobium*. Wave length, amplitude and pitch angle are for attached flagella.

Strain	Helicity	Wave length (μm)	Length of flagella (μm)		Amplitude (μm)	Pitch angle (deg)
			Attached	Super		
wild type	RH	1.00 $\pm$ 0.02	3.85 $\pm$ 0.10	22 $\pm$ 2	0.36 $\pm$ 0.09	35 $\pm$ 2
1-07	RH	1.06 $\pm$ 0.03	3.90 $\pm$ 0.10	24 $\pm$ 2	0.42 $\pm$ 0.04	37 $\pm$ 2
1-12	RH	1.04 $\pm$ 0.02	3.85 $\pm$ 0.02	36 $\pm$ 2	0.42 $\pm$ 0.03	38 $\pm$ 2

Helical parameters of halobacterial flagellar polymorphs

Type	Handedness	NaCl in solution (M)	pH	Wavelength (μm)	Amplitude (μm)	Pitch angle (°)
Normal	RH	4	7	1.16 $\pm$ 0.04	0.4 $\pm$ 0.03	38 $\pm$ 2
Curly	RH	4	9.2-9.5	0.35 $\pm$ 0.02	0.30 $\pm$ 0.04	61 $\pm$ 2
Normal	ND	0.8	7	1.02 $\pm$ 0.02	0.33 $\pm$ 0.02	35 $\pm$ 2
Normal	ND	0.1	7	1.20 $\pm$ 0.05	0.52 $\pm$ 0.02	40 $\pm$ 2
Mixed (ring and curly)	RH (curly)	4	10.5	—	0.85 $\pm$ 0.05	—
Mixed (normal and straight heat treatment)	RH	4	7	1.27 $\pm$ 0.02	(ring diameter) 0.48 $\pm$ 0.02	34 $\pm$ 3

3.5 Production of antibodies and behaviour of tethered cells

3.5.1 Halobacteria

Because antibodies produced in rabbits and mice are inactive at the high salt concentration (4 M-NaCl) necessary for the stability of halobacterial flagella, I used chicken antibodies, which are active at higher salt concentrations (Goodman et al., 1950).

The flagellar antibodies were used to tether cells to microscope slides or cover slips according to Silverman and Simon (1974). I observed flagella of two, three or more cells connected to each other, which demonstrates that flagella from different cells were cross-linked by the antibodies (Fig. 24). In many cases, one cell rotated around its flagellar bundles while the rest of the clumped cells remained stationary. When a single halobacterial cell was tethered, the cell body rotated in the CW or CCW direction, and during the change of rotation from CW to CCW or vice versa the cell always stopped (Fig. 25). This results proves that the motility of halobacteria is due to the function of a rotatory unit. The rotatory unit could consist of a single motor to which one or all filaments are attached or could consist of individual motors for each filaments.

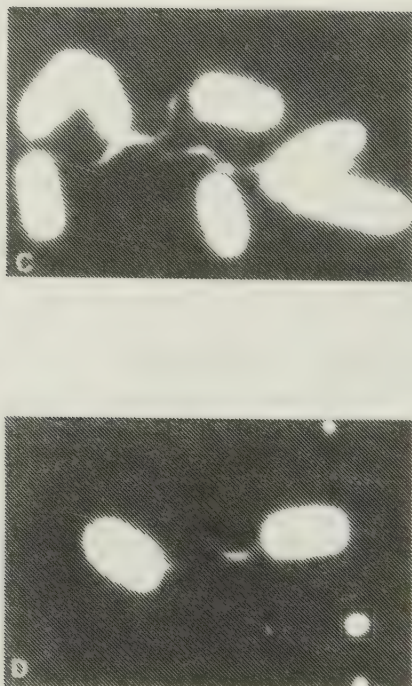


Figure 24

*Dark-field micrographs of tethered halobacterial cells.
(a) and (b) Flagellar bundles of several halobacterial cells
are cross-linked by antibodies.*

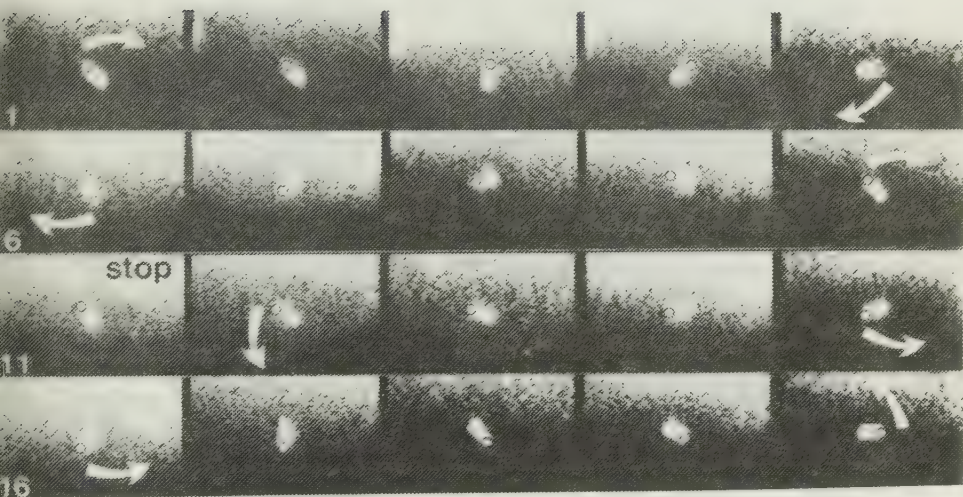


Figure 25

Motility behaviour of a single tethered halobacterial cell. The photographs were taken from a video film at an intervals of 0.2 sec. The tethered cell is shown rotating clockwise, stopping and then turning counterclockwise. The arrows indicate the direction of rotation of the cell body marked by circles.

3.5.2 Square bacteria

If the square bacterial cell body or the flagella are immobilized by tethering, the other part still can rotate, thus demonstrating that like in halobacteria a rotatory unit exists between the cell body and flagellar bundles. Interestingly, the rotational wave form flagellar bundles always remain stationary. Fig. 26 demonstrates a square bacterial cell tethered by its flagellar bundle and rotating around its square cell body. In cell suspensions, cells clumped by their flagella occur quite often and are responsible for the apparant tumbling seen under the microscope. These cells can be of the type shown in Figure 14C and be attached to the clump by one filament while the other filament remains free. Thus, these cells may produce the kind of motility known, from uncoordinated eubacterial flagellar rotation, as tumbling. The multiple attachment of filaments or bundles to various points of the cell surface, producing a rotating giant flagellar bundle, causes compensatory rotation of the cell body during translational movement.

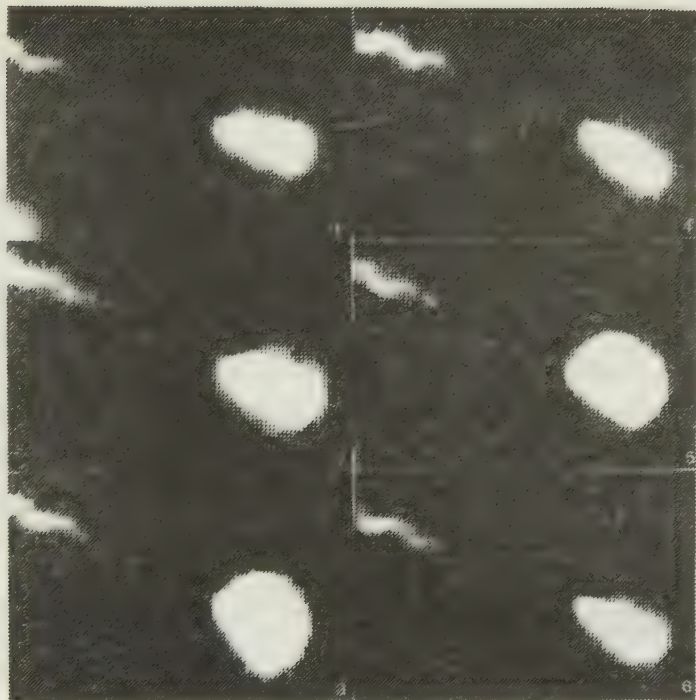


Figure 26

*Cell body rotation of tethered square bacterial cells.
The photographs were taken from a video film.*

3.6 Excitation signal processing time in phototaxis

3.6.1 Attractant photosensing system

Cells adapted to continuous orange-red light (600 ± 20 nm) respond to a decrease in the light as unfavorable stimulus and reverse their swimming direction. The reversal frequency of a population of cells was monitored during such step-down attractant light stimuli delivered at $t = 1$ sec in Fig. 27. There is a clearly resolved latency period after stimulation in which the population reversal frequency is unchanged from its prestimulus value, followed by an increase in reversal frequency (excitation) and subsequent return to its prestimulus value (adaptation). For the largest stimulus in Fig. 27 (circles), the minimum latency period was 0.70 ± 0.07 sec. The remaining curves indicate reversal distributions for stimuli of decreasing relative intensity. The distributions are broadened and shifted toward later times, with approximately the same minimum latency (earliest detectable increase over background, ~ 0.7 sec). The maximum light intensity used (7.8×10^4 ergs/cm² per sec) was sufficient to saturate the response (inset). The fraction of cells reversing in the 5-sec period saturates with a similar stimulus-dependence as the time to peak reversal frequency.

3.6.2 Repellent photosensing system

Reversal distributions after step-increase in repellent light (effective bandpass: 360-520 nm; $I_{\max}$, 3.9×10^5 ergs/cm² per sec) are shown in Fig. 28. The distributions are sharper, with a minimum latency of 0.31 ± 0.10 sec. and a peak at 0.76 ± 0.14 sec for the most intense stimulus (circles). Again, lowering the stimulus intensity resulted in broadened, shifted distributions. As with attractant light, repellent light stimuli generate a family of distributions with mean latency decreasing with increasing stimulus strength and with a characteristic minimum latency. The family of distributions characterizing the broad-band repellent response differs from that of the attractant response both in terms of the apparent minimum latency and in the relationship between mean latency and fraction of population undergoing stimulus-induced reversals.

3.6.3 Discrimination of two distinct repellent photosystems

Previously, it has been reported that repellent sensitivity at ~400 nm is dependent on the presence of red background illumination (Spudich et al., 1984; Takahashi et al., 1985), as expected from the proposal that a blue-absorbing intermediate of the SR molecule acts as a repellent receptor (Spudich et al., 1984). The 529-nm shortpass filter used in the experiments described above was chosen because it delivers a broad-band light, which overlaps the absorption spectra of both the SR ground-state

SR₅₈₇) and its blue-absorbing photointermediate, S₃₇₃. In view of a recent report (Takahashi et al., 1985) describing an additional repellent sensitivity at longer wavelengths than absorbed by S₃₇₃, we examined the repellent-stimulus latency using more sharply defined narrow-band filters.

Fig. 29 shows the reversal distributions generated by delivering 400 nm light stimuli in the presence and absence of orange-red background light (600 ± 20 nm). There was a clear increase in the response in the presence of the background light, with a minimum latency of 0.70 ± 0.14 sec. The response to 450 nm light is shown in Fig. 30. There is a peak in reversal frequency at ~ 0.84 sec, with a minimum latency of 0.40 ± 0.07 sec. Unlike the 400-nm stimulus, the response showed little or no dependence on red background illumination.

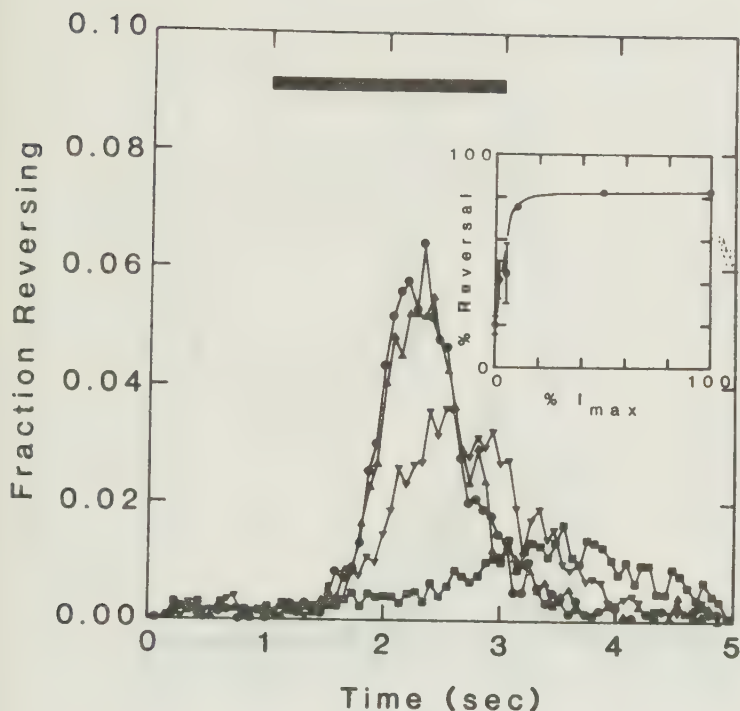


Figure 27

Reversal distributions after step-decreases in attractant light. Stimulus, 600 ± 20 nm; I_{max} , 7.8×10^4 ergs/cm² per s. Circles, maximum intensity; Δ , 50% relative intensity; two independent data sets; 1,410 reversals total. Triangles, 10% relative intensity; two independent data sets; 1,437 reversals total. Inverted triangles, 5% relative intensity; three independent data sets; 1,294 reversals total. The curves have been normalized to sample size and fraction of cells reversing. The bar indicates the duration of the stimulus. Data was collected for 40 5-s video segments at 30-s intervals. Inset, dose-response curve derived from the data shown in the main figure.

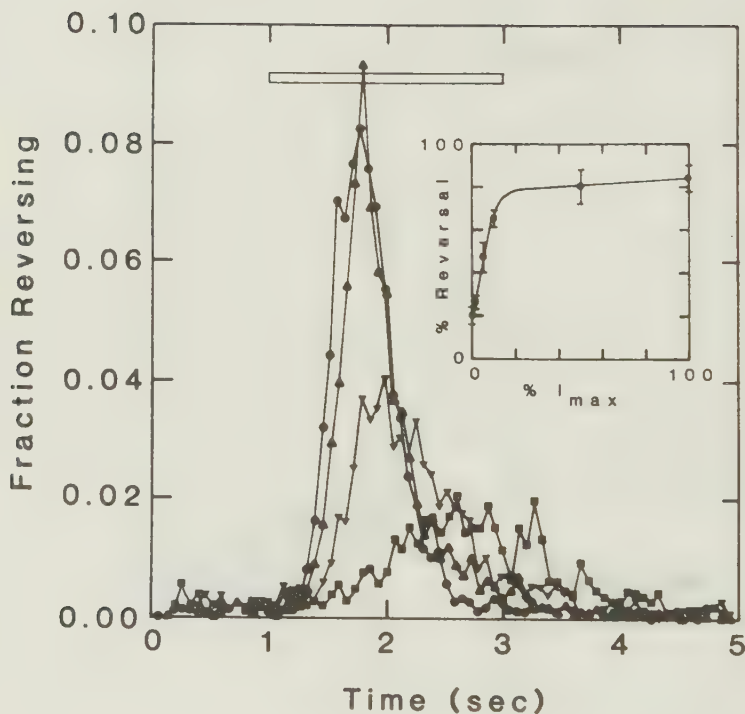


Figure 28

Reversal distributions after step-increases in repellent light. Stimulus, 520 nm short-pass; I , 3.9×10^5 ergs/cm² per sec. Circles, maximum intensity; three independent data sets; 2,000 reversals total. Triangles, 50% relative intensity; two independent data sets; 1,327 reversals total. Inverted triangles, 10% relative intensity; two independent data sets; 1,155 reversals total. Squares, 5% relative intensity; two independent data sets; 497 reversals total. The bar indicates the duration of the stimulus. Inset, dose-response curve derived from the data shown in the main figure.

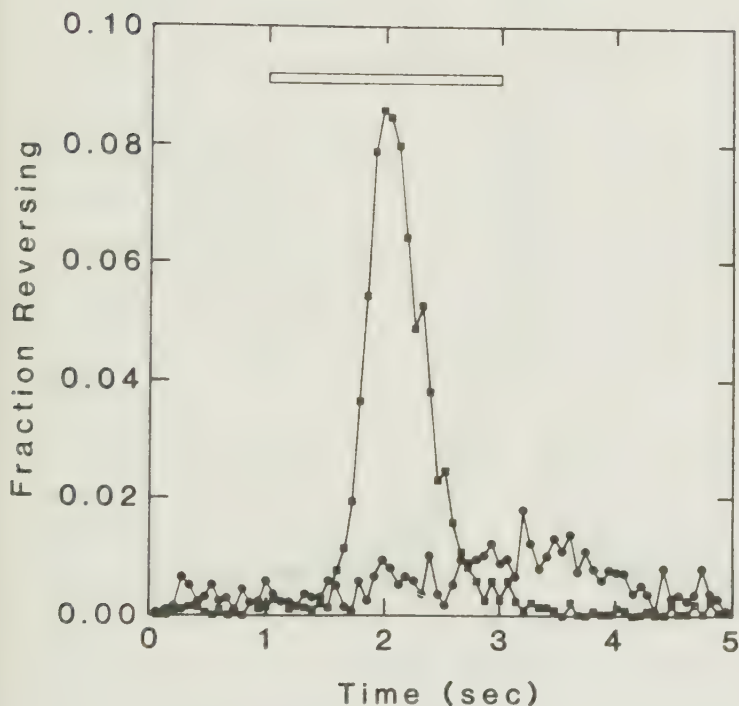


Figure 29

Reversal distributions after a step-increase in 400 nm light. Stimulus, 400 ± 5 nm light; I_{stim} , 4.0×10^4 ergs/cm² per sec. Circles, infrared background illumination ($\lambda > 700$ nm; 2.8×10^4 ergs/cm² per sec); two independent data sets; 570 reversals total; 41% reversing within the 5 sec period. Squares, infrared plus orange-red (600 ± 20 nm, 4.9×10^4 ergs/cm² per sec) background illumination; two independent data sets; 1,499 reversals total; 83% reversing; minimum

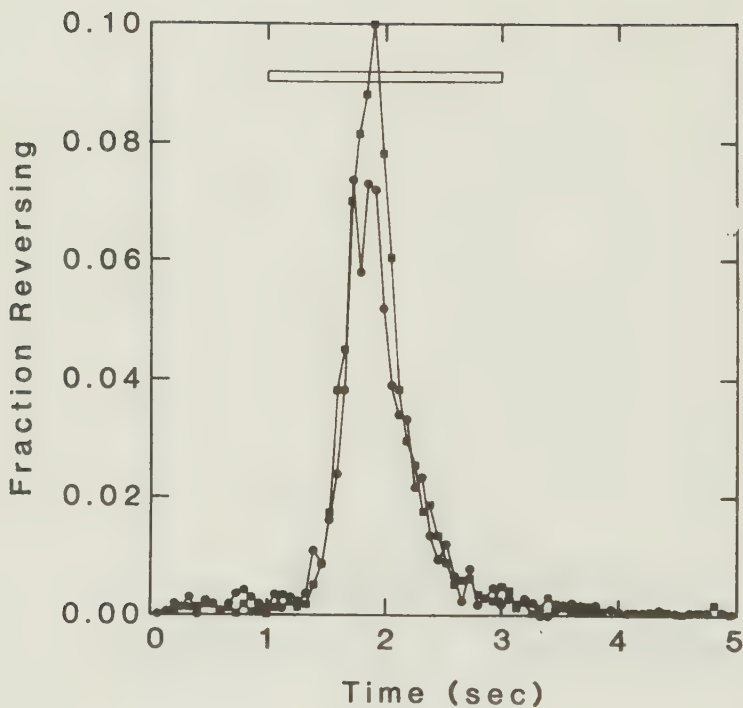


Figure 30

Reversal distributions after a step-increase in 450 nm light. Stimulus, 450 ± 20 nm; I_{max} , 3.5×10^4 ergs/cm² per sec. Circles, infrared background illumination as in fig. 29; two independent data sets; 1,164 reversals total; Squares, infrared plus orange-red background illumination as in fig. 29; two independent data sets; 1,425 reversals total; 82% reversals. The bar indicates the duration of the stimulus.

3.7 Transducer Proteins

3.7.1 Visualization of methyl-accepting proteins(transducers) on gels

The whole cells containing radiolabeled methylated proteins and lipids can be processed directly for electrophoresis and each methylated component visualized as an isolated entity (a radioactive band) by fluorography or autoradiography of dried gels. This procedure has been utilized for extensive characterization of transducer molecules as radiochemically pure species (Hazelbauer, 1984).

Methyl-accepting proteins in *Halobacterium halobium* appeared on SDS-polyacrylamide gel as a series of ^3H -methyl labeled bands with apparent molecular weights in the region of 90-125 kilodaltons and in the region of 20-25 kilodaltons (Fig. 31A and C) as deduced from the position of the markers on the gel. Each of the four transducer proteins in eubacteria has a molecular weight near 60,000 daltons and the multiple forms of the various proteins in a wild-type cell create a complex, overlapping pattern of methyl-labeled bands on gels (Boyd and Simon, 1980; 1983). Reducing of the bisacrylamide concentration from 0,8% to 0.2% resulted in a gel with greater porosity and halobacterial methyl-accepting proteins were more widely separated in low bis gel (Fig. 31C and D). A *Halobacterium halobium* mutant isolated as defective in chemotaxis exhibit greatly reduced levels of

these methyl-labeled proteins (Fig. 31B and D).

3.7.2 Steady-state level of methylation and methyl group turnover

To observe the kinetics of methyl-exchange in unstimulated cells I carried out time course experiment stopping *in vivo* labeling at various times during 80 minutes incubation with L-(methyl-³H-methionine (Fig. 32), which showed that after approximately 60 minutes a steady-state level is reached.

Labeling with L(methyl-³H)- methionine) for 80 minutes followed by a chase with 100mM of non-labeled methionine for 20 to 80 minutes showed that turnover of methyl groups occurs mainly on the upper methylated bands (Fig. 32). Kinetics of the turnover of lower methylated bands were slower.

3.7.3 Loss of radiolabeled carboxymethyl esters by base-catalyzed hydrolysis

Eubacterial chemotaxis receptors are reversibly methyl esterified upon binding of chemoattractants. This methylation of the γ -carboxyl of glutamic acid can be hydrolyzed, either chemically or enzymatically, producing the original carboxylic acid residue and methanol (Van Der Werf and Koshland, 1977; Kleene et al ,1977). I also carried out a diffusion assay which distinguishes ³H-methionine from

the base-labile ^3H -methylesters. Treatment of methyl-labeled samples with 0,5M NaOH removed methyl groups from the methyl-accepting proteins (fig. 33); shown is the region of the gel containing the methyl-esterified halobacterial proteins which appear as a narrow peak of base-labile radioactivity. This is clear evidence for carboxyl methyl ester linkage, which is analogous to the eubacterial transducers. When labeling was carried out while inhibiting the protein synthesis by puromycin base-labile radioactivity was higher at 20 -25 kd region. Thus lower methylated bands might either be the product of degradation of upper bands or some intermediate methyl acceptor.

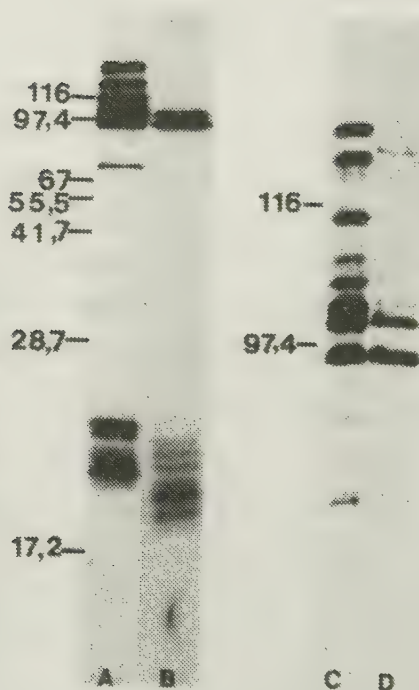


Figure 31

Fluorogram of SDS-PAGE of radiolabeled methyl-accepting proteins. Experiments were performed as described under "Material and Methods". Lane A: wildtype strain Flx15, Lane B: Chemotaxis mutant M402. Lane A and B were from 14% PAGE. Lane C: and D were from 11% PAGE but having 0.2% of bis acrylamide. Marker proteins were indicated (Kilodalton) respectively.

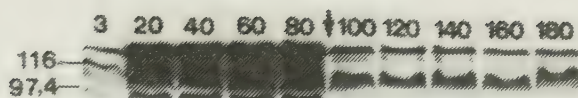


Figure 32

Steady-state level of methylation and methyl group turnover. Experiments were performed as described under "Materials and Methods". Numbers indicate time in minutes. Arrow shows when 1mM cold L-Methionine was added to the cell suspension.

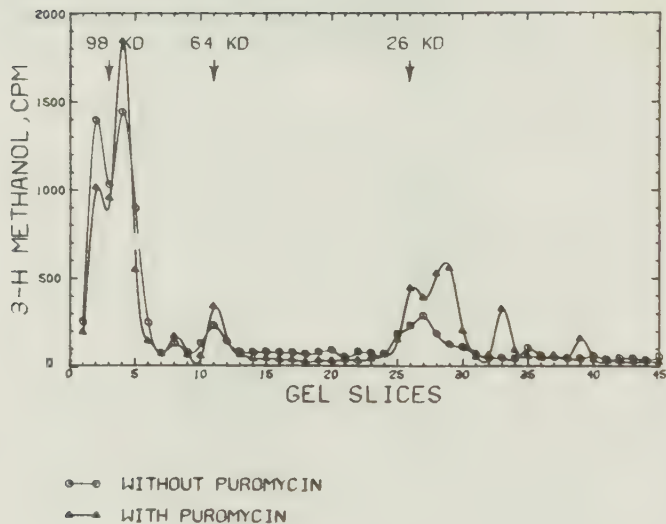


Figure 33

Comparison of volatile/base-labile radioactivity, derived from protein-methyl esters with and without the inhibition of protein synthesis by puromycin. The arrows indicate the position of protein markers.

3.7.4 Change in methylation upon addition of chemical stimuli

Application of a mixture of attractants L-histidine and L-leucine to labeled halobacterial cells decreased the intensity of labeling of heavy polypeptides (Fig. 34B). Highest decrease was shown when peptone was added (Fig. 34C). However, these results were not always reproducible. It is interesting to note that in *B.subtilis* attractants also caused the demethylation of methyl-accepting proteins.

The activities of two chemotaxis-specific enzymes, a methyltransferase and a methylesterase, are regulated during adaptation to maintain behavioral responsiveness. To monitor stimulus-induced changes in methylesterase activity in intact halobacterial cells, we quantitated the continuous generation of radioactive methanol, the end product of the demethylation reaction, in a flow device according to (Kehry et al, 1985). The addition of 10mM mixture of attractants L-histidine and L-leucine to wild-type cells resulted in a 4 fold increase in the rate of ^3H -methanol production from the prestimulus rate in fig. 35A. The largest effect was seen with peptone (Fig. 35B), which is the strongest attractant for *halobacterium halobium*. The direction of the attractant-induced changes in methanol production were also interesting. It appeared that a transient increase was seen in methanol production when attractant is either added or removed. In general, adaptation from the first spike (caused by attractant addition) was more rapid than recovery from

the second spike (caused by the attractant removal). The double spike phenomenon was observed with each of attractants to which response was detectable. To further analyze the methylesterase activity I added the repellent phenol to wild-type cells. Fig 35C showed that addition of repellent 5mM phenol also caused increase of methylesterase activity. Thus in *Halobacterium halobium* both positive and negative chemical stimuli caused the rapid evolution of methanol, in contrast to eubacteria.

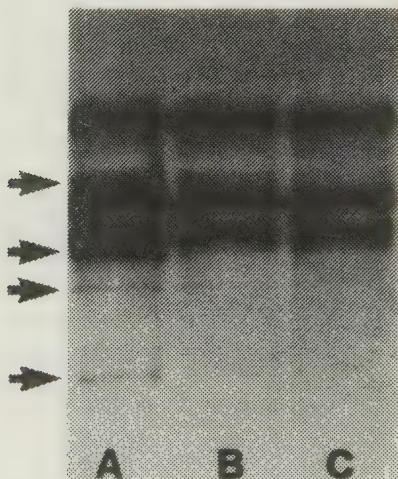


Figure 34

Effect of of attractants on MCP methylation. Experiments were performed as described under "Materials and Methods". Lane A; Buffer control; Lane B: 10mM of Histidine and 5 mM of L-leucin; Lane C: 0.1% of oxoid peptone. Arrow indicated the bands where intensive demethylation occurred.

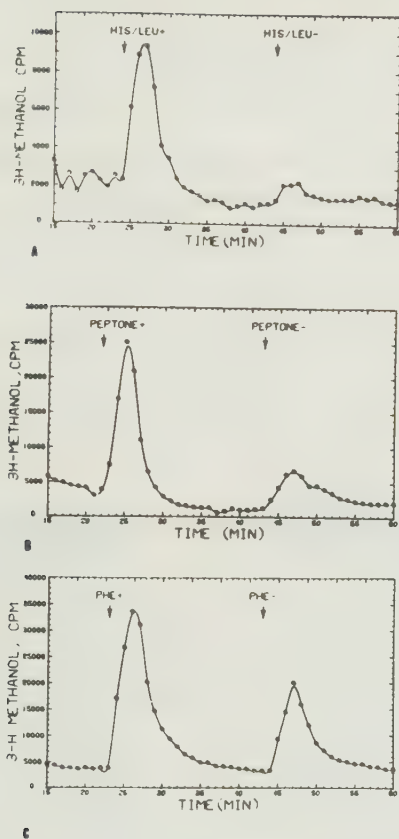


Figure 35

Flow assay of methanol evolution: effect of addition and removal of chemoattractant and repellent. Cells were labeled as described under "Materials and Methods". Labeled cells were applied to the filter and then flushed with chase medium. Arrows indicated the addition or removal of chemoeffectors. Fig A: Attractants, 10mM histidine and 5mM of L-leucine; Fig B: Attractant, 0.1% of oxoid peptone; Fig C: repellent phenol (5mM). The fraction collecting was 1 min, flow rate was 0.4ml/min.

3.7.5 Change of methylation upon photostimuli

The relative intensity of label in the light polypeptide increased after stimulation of cells with attractant light ($587 \pm 5\text{nm}$) (Fig.36B) or with blue light for 20 minutes. This phenomenon was also reported by Schimz and Bibikov (Schimz., 1981 and 1982). The important point to note here is that the profile of volatile/base-labile radioactivity of lower molecular weight bands was relatively low when protein synthesis was not inhibited with puromycin (fig. 33). Thus the stability of low molecular weight polypeptide of halobacterial transducer proteins depends upon protein synthesis. It might be relevant to reports of a low $M_r(19000)$ band associated with *B.subtilis* chemotaxis (Ullah. A. and Ordal, 1981). In *B.subtilis*, however, methylation of the 19000 molecular weight protein was not modified by chemoeffector stimuli. To answer the question whether lower methylated proteins are involved in phototaxis I did the same experiment with green and blue light stimuli in a retinal deficient mutant L-07. In this case light stimuli can not be processed because the lack of photoreceptor. Fig. 36A(Lanes 2 and 3) demonstrated that there was a increase in the relative intensity in the lower as well as in the upper methylated bands. Thus it seems that low molecular weight polypeptide is not related to the phototaxis signal pathway. I also carried out flow-chase experiments upon stimulating cells with green or blue light. Fig. 37A shows that as in chemotaxis both positive and negative photostimuli increase the methanol production and after a certain time it returns

to its prestimulus level. If *H. halobium* responds to both positive and negative photostimuli by demethylating its transducers and by augmenting the evolution of methanol then a phototaxis deficient but chemotaxis positive mutant must not release methanol after illuminating with photostimuli. Fig. 37B and C demonstrated that mutant *pho81* did not released methanol after illumination of photostimuli but increased the methanol formation when chemoattractant peptone was added and removed. Thus as in chemotaxis, demethylation of transducers and evolution of methanol also affords adaptation in phototaxis. The time of adaptation in phototaxis was more rapid (seconds) than in chemotaxis (minutes)

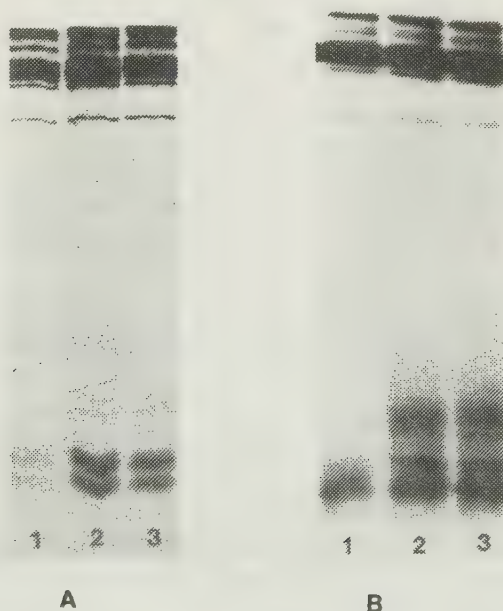


Figure 36

Effect of blue and green light on MCP methylation A:
 lane 1, dark control; lane 2, blue light ($375 \pm 5\text{nm}$) was delivered for 20 minutes; lane 3, green light ($585 \pm 5\text{nm}$) was delivered for 20 minutes. These experiments *IN VIVO* were performed using strain L07, a retinal deficient mutant. B:
 The same experiments were carried as in (A) but only with wildtype cells S-9.

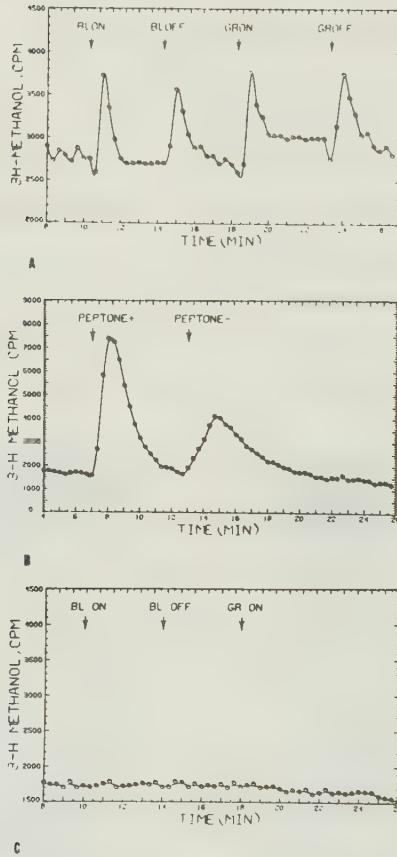


Figure 37

Flow assay of methanol evolution during positive and negative photostimuli. Experiments were performed as in fig. 35 except the flow rate (1.5 ml/min) and the fraction collecting time (18 seconds). Fig A: Strain Flx15, blue and green light as in Fig. 36. Fig B and C: mutant pho81.

DISCUSSION

4.1 Right-handedness of Halo- and square bacterial flagella

Halobacterium halobium has two types of flagellar formation, monopolar and bipolar bundles, and non-flagellated cells were also observed. The ratio of these forms changes during growth. This could be easily explained by an independence of cell division and the synthesis of the flagella. In contrast to this, the cell cycle and flagella formation in eubacteria seem to be correlated (Iino, 1977).

Square bacterium has no definite type of flagellar formation. It varies from mono to peritrichous. The giant flagellar bundle can form from different single filaments, from single filaments and bundles, and from different bundles. The multiple attachment of filaments or bundles to various points of the cell surface, producing a rotating giant flagellar bundle, causes compensatory rotation of the cell body during translational movement.

Both Halo- and square bacteria have a right-handed helix in contrast to the left-handed helix found in most eubacteria (Asakura et al., 1966; Götz, 1982) which, however, can be changed to the right-handed form by mutation, pH or ionic strength changes, viscosity, etc. (Macnab & Ornston, 1977). One might argue that extremely high salt concentrations at which Halo- and square bacteria

live induce the right-handed helical form of the flagella. But in low salt solutions filaments have the same form as in high salt. Thus polymorphic transition of these flagella induced by ionic strength can be ruled out. These properties may be a common physiological feature of archaebacteria.

4.2 Flagellar bundle never flies apart

Peritrichous eubacteria are propelled by several filaments arising at random points on the surface of the cell. Each filament is powered by a rotatory motor at its base (Berg & Anderson, 1973; Silverman & Simon, 1974). When the motors turn CCW, the filaments form a bundle and the cell swims; when the motors turn CW, the bundle flies apart and the cell tumbles (Larson et al., 1974; Macnab & Ornston, 1977). In *Halobacterium halobium*, CW rotation of the right-handed helical flagella exerts a pushing force on the cell body and CCW rotation pulls the cell, and the bundle never flies apart. The same is true for square bacteria. The strong lateral affinity which these flagella display is presumably responsible for the ability to undergo negative wave propagation without jamming during backward swimming, thus maintaining the phase cohesion. This is true for halobacteria but not for peritrichous square bacteria.

Studies of the motility behaviour of tethered cells demonstrated clearly that both halo- and square bacterial flagellar bundles are powered by rotatory motors. The antibodies used for the tethering could: (1) connect a

single flagellum of a bundle to flagella of other cells; (2) crosslink the filaments within the flagellar bundle; or (3) mediate connection of a flagellar bundle or a single longest flagellum to the glass surface. It is possible that a cell is tethered by two different filaments which make it rotate, but only if the motors have equal and opposite velocity. Rotation by three or more tethered filaments is impossible because there is no way that angular velocity of different filaments will cancel each other. The antibody experiments independent of the type of crosslink pose an interesting question: whether the rotatory unit consists of a single motor to which one or all filaments are attached or could consists of individual motors for each filament. The tethering experiments are not conclusive evidence for a rotating plate. The rotation of a cell around its axis, when tethered to another cell's bundle or to a glass surface, could be explained by only one flagellum of the bundle actually being tethered. The question is how then the waveform of the remainder can remain stationary? This could be explained in two ways: 1) conformational phase change of remaining filaments would produce a stationary waveform in spite of their rotation at angular velocity of the cell body; 2) rotation of the flagellar motors in the bundle at negative angular velocity of the cell body. Both of these mechanism could produce a net phase velocity of the non-tethered filaments of zero. Presumably, a strong lateral affinity among the non-tethered flagella would ensure a common phase velocity, and if they also interact tightly

with the tethered flagellum this common phase velocity would be zero. In the conformation explanation, the non-tethered filaments are rotating with regard to the tethered one, and in the rotation explanation they are also rotating with regard to each other. Further electron microscopy of flagellar motor will be required to resolve this question.

The cell wall structure in halobacteria is quite different from the murein-based cell walls of eubacteria (Blaurock et al., 1976), because only a thin protein layer covers the cell membrane. The mechanical anchoring of several rotatory motors similar to flagellar basal body of eubacteria presents a problem. One could assume that several tightly coupled several single filaments as seen in Fig. 45 might extend through the periplasmic space and the cell membrane into a cytoplasmic structure, which serves the purpose of mechanical stabilization of the rotors.

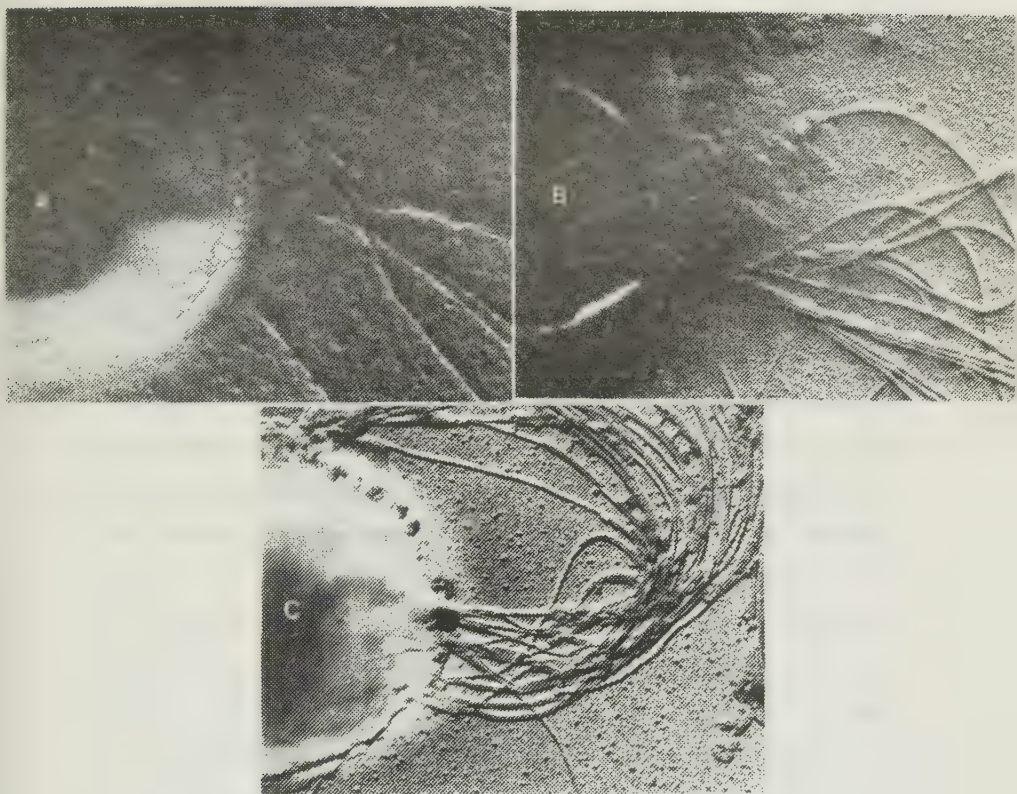


Figure 38

Electronmicrographs of H.halobium showing the position of attachment of flagellar bundle to the cell body surface.

4.3 Three flagellin-proteins in *Halobacteria* and only one in *Square bacteria*

In eubacteria, pure flagellin is coded for by a single gene and SDS-PAGE shows a single band, except in *Caulobacter*, where two types of flagellin are coded for by two genes (Gill & Agabian, 1982). Purified halobacterial flagella by dissociation and reconstitution showed that flagellar filaments consist of three proteins: FlaI, FlaII and FlaIII. In contrast, square bacterial filaments are from one protein. Upon reconstitution of the flagella in *H.halobium* the three proteins retain their multiple-band appearance. This heterogeneity is due to the fact that halobacterial flagellins are sulphated glycoproteins with varying degrees of glycosylation (Wieland et al., 1985). The sulfated saccharides are linked via glucose to asparagine residues in the individual flagellins. Interestingly, the apparent molecular weight of the three flagellins is shifted towards lower values in strain M-175 compared to the parent strain L-33. This can be taken as an indication of incomplete glycosylation of the proteins, resulting in defective flagellar structure and causing release of flagellins to the medium and continuous biosynthesis of the flagellins (Wielands et al., 1985). However, the different degrees of glycosylation in the mutant M-175 and parent strain L-33 do not cause different behaviour of the flagellins during dissociation and reconstitution of the flagella.

4.4 Excitation signal processing times in phototaxis

Using a commercial digitizer-based motion analysis system coupled to an electronic shutter controlling delivery of light stimuli we have measured excitation signal processing times in the phototaxis system of *H. halobium*. Automated analysis of rates of change of direction and linear speeds provided detection of swimming reversals with 67-ms resolution, permitting measurement of both the rapid excitation phase and slower adaptation phase of responses to attractant and repellent photostimuli. The peak response latencies reported here are comparable to the mean latencies of 1-2 s for saturating stimuli measured by visual tracing, and increase with decreasing stimulus strength (Dencher., 1978; Dencher & Hildebrand., 1979; Schimz & Hildebrand., 1985). The timescale of the peak response, 0.76-1.34 s, is slower than the 0.20-s mean latency for chemotaxis signalling in *E. coli* measured by Segall et al (1982).

The reversal distributions induced by step-decreases in 600 nm light and step-increases in 400 nm light had approximately the same minimum latency of 0.70 s but different peak times. Reversal induction at these wavelengths and the orange-red light-dependence of the 400 nm response are expected from the properties of the SR attractant form ($\lambda_{\max}$, 587 nm) and its repellent photointermediate S_{373} ($\lambda_{\max}$, 373 nm) (Bogomolni & Spudich., 1982; Spudich & Bogomolni., 1984).

Takahashi et al (1985) have recently observed repellent

photosensing with peak sensitivity between 450 and 520 nm in a mutant strain Flx3-12 closely related to Flx15, and report the detection of a flash-induced absorbance change in this region that has different kinetics from that due to SR photocycling. They have suggested that a retinal pigment other than SR mediates this repellent response. The response to 450 nm light is independent of orange-red background illumination, which indicates that the receptor mediating this response is not an SR photointermediate.

Recently Marwan & Oesterhelt (1987) demonstrated that light around 530 nm causes a photophobic response; without green background light. The action spectrum of this background light-independent response has a maximum at about 480 nm and they call the new pigment P_{480} . According to their hypothesis both photoreceptors (SR and P_{480}) feed into a common signal chain, which consists of a photocatalysed reaction producing a signal (Wolfgang & Oesterhelt., 1987). However the experimental results presented here shows that the reversal distribution after a step-increase in 450 nm light differed from that for the 400 nm light in that the apparent minimum latency was shorter, 0.40 s compared with 0.70 s for the SR-mediated response under the conditions used for the two experiments.

Methyl-accepting taxis proteins in Halobacteria

Transducers or so called methyl-accepting proteins (MCP) of halobacteria differ from eubacteria in relation to the

molecular weight. In eubacteria all four MCPs are 60 kilodalton proteins. In halobacteria there are two types: 90-125 and 20-25 kilodalton proteins. The appearance of 20-25 kd methylated proteins varies on the inhibition of protein synthesis. Thus it may be the degradation product of the high molecular weight MCPs or some methyl-accepting intermediate of cell metabolism. Both high and low molecular weight proteins release methyl groups by base treatment, demonstrating an ester linkage in analogy to the eubacterial transducers. The absence of most of the methylated proteins in chemo- and phototaxis deficient mutant demonstrate that these methylated proteins are related to the behavioural signal pathway. The positive and negative chemical and photo stimuli cause a rapid production of methanol with gradual return to the prestimulus level. Thus it seems that adaptation in both chemo- and phototaxis is controlled by demethylation.

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